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CLINICAL AND EXPERIMENTAL

THE MACROPOLYCYTE*

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THE statement that megakaryocytes are occasionally found in the peripheral blood has been repeated in textbooks and monographs during the past thirty years, but I have never seen in blood films a cell which faithfully resembled the multinucleated giant cell of the marrow; nor have I seen photomicrographs or drawings of the cells so described that are at all convincing.

There is every probability that these so-called megakaryocytes belong to one or other class of macropolycytes.

DEFINITION

The normal neutrophile polymorphonuclear leucocyte, for brevity called the "polymorph," measures, in fixed and stained films 10 to 12 and occasionally up to 14 micra in diameter. In certain conditions giant polymorphs measuring 16 to 20 or more micra in diameter are found, to which the descriptive title "Macropolycyte" is given (1927).

Morphologically there are three types of macropolycytes. The first or Type 1 resembles the other polymorphs in the blood film in that its nuclear structure and fine oxyphil cytoplasmic granules are indistinguishable from those in the polymorph. The only differences from that cell are the large size and frequent hypersegmentation of the nucleus.

The Type I macropolycyte is found: (1) in health; (2) in infections and intoxications where there is bonemarrow reaction, e.g., the acute pyogenic

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infections, tuberculosis, malaria, the exanthemas, carcinoma, myeloid leucemia, and in rabbits with experimental anemia due to ricin or repeated hemorrhages; (3) pernicious anemia.

The Type I macropolycyte is illustrated in Plate II, Figs. 2 to 7, and in Plate III, Figs. 9, 10, 11, 12, 13, and 14.

The Type II macropolycyte resembles the megakaryocyte of the marrow in that its nucleus is gnarled and somewhat condensed and may be of the simple horseshoe type, or may have as many as 14 segments. The cytoplasmic granules are coarse and are not all oxyphil as in the polymorph, but azurophil granules are present in varying numbers.

The cell is found in pernicious anemia and is illustrated in Plate III, Figs. 15 to 25.

The third type of macropolycyte has features of both the preceding types. It resembles the Type I cell in having fine oxyphil granules in the cytoplasm, while the general nuclear conformation is that of Type II. It differs from both in nuclear structure. As the figures on Plate IV show, the nuclear structure is a very open meshwork suggesting a deficiency in basichromatin, and the bulk of the nucleus appears to be large in comparison to the cytoplasm. The nucleus is often rolled and folded on itself, but hypersegmentation is not frequently seen. This type like Type II occurs in pernicious anemia.

THE POLYMORPH IN HEALTH AND IN INFECTIONS

For the better appreciation of the macropolycyte a few remarks on the polymorph are necessary. The polymorph enters the blood stream from the marrow with a single-lobed nucleus, and as its life in the circulation continues, the nucleus becomes more lobulated until toward the end of its existence it has four or five segments united by fine strands of chromatin (Plate I). In the blood stream, therefore, there are, normally, cells representing the various periods from youth to senility, and it was found by Arneth (1904) that the numbers of polymorphs with one, two, three, four, or five nuclear segments were remarkably constant in health and could be classified on those lines. Our average normal count, that is the percentage of cells in the various classes corresponding to nuclear segments, is:

Class	I	II	III	IV	V
	10	25	47	16	2

This was the average of the counts of 90 normal persons of both sexes between the ages of twelve and fifty-five years. The greatest possible care was taken to exclude infection at the time the blood was taken, and for two months previously. The teeth, throat, nose, ears, chest, abdomen, and urine were examined, and in many cases radiograms were taken of the chest and teeth.

The length of life of the polymorph in the blood stream has been estimated on experimental and clinical grounds (Ponder, 1926) (Cooke and Ponder, 1927) to be approximately three weeks. The figures given above represent a polymorph population comparable to infants, children, men, old men, and senile men, in which the deaths in the older groups are exactly compensated for by the births from the marrow. The figures of the polynuclear count tell us

whenever this normal state becomes altered, whether by the addition of an unusually large number of young cells, by the removal of an unusually large number of old cells, by both these conditions combined, or by some interference with the normal development.

It will be noted that no class is provided for cells with more than five nuclear segments. Such polymorphs are rare in normal blood and when they do occur they are placed in Class V.

In infective conditions the percentage of cells of Classes I and II is increased, and the older cells of Classes IV and V rapidly disappear from the circulation. The count has, therefore, a left-handed drift, as in the following examples:

	I	II	III	IV	V
Normal	10	25	47	16	2
Typhoid fever	54	31	15	–	–
Scarlet fever	44	43	13	–	–
Measles	45	39	16	–	–
Erysipelas	45	40	14	1	1
Diphtheria	41	39	17	3	–
Rubella	30	35	32	3	–
Varicella	33	35	28	4	–
Pertussis	34	39	27	–	–
Puerperal sepsis	42	37	21	–	–
	I	II	III	IV	V
Cerebrospinal fever	40	45	14	1	–
Lobar pneumonia	57	26	15	2	–

These facts make the appearance of the macropolycyte the more remarkable, because, although a fairly rare cell, it is found in infections frequently associated with a polymorphic leucocytosis and always with a left-handed polynuclear count.

Before proceeding to further discussion, the moment is opportune to suggest that, although the Type I macropolycyte found in pernicious anemia may be morphologically identical with its prototype found in health and in intoxications, the cause of its appearance is probably very different. For this reason the Type I macropolycytes of health and of intoxications would be better considered together, and the types found in pernicious anemia classed under the heading "the macropolycytes of pernicious anemia."

THE TYPE I MACROPOLYCYTE IN HEALTH AND IN CONDITIONS OF ABNORMAL MARROW ACTIVITY

1. *In Health.*—Macropolycytes are extremely rare in health, no example being seen in the blood films of the 90 investigated normal persons that formed the basis of our average normal count. Recently, however, I observed a macropolycyte in a man, aged thirty-four years, whose polynuclear count had previously been included in the original 90. He was reinvestigated, but no focus of infection could be found. The counts of the two occasions were almost identical:

I	II	III	IV	V
11	31	42	14	2

The count has a weighted mean of 2.65, which falls close to the average mean of 2.74, and within the limits of normality. I think we must accept the fact that macropolycytes do very occasionally appear in normal blood films.

2. *In Conditions of Abnormal Marrow Activity.*—This type is illustrated by the Figs. 2 to 7 in Plate II.

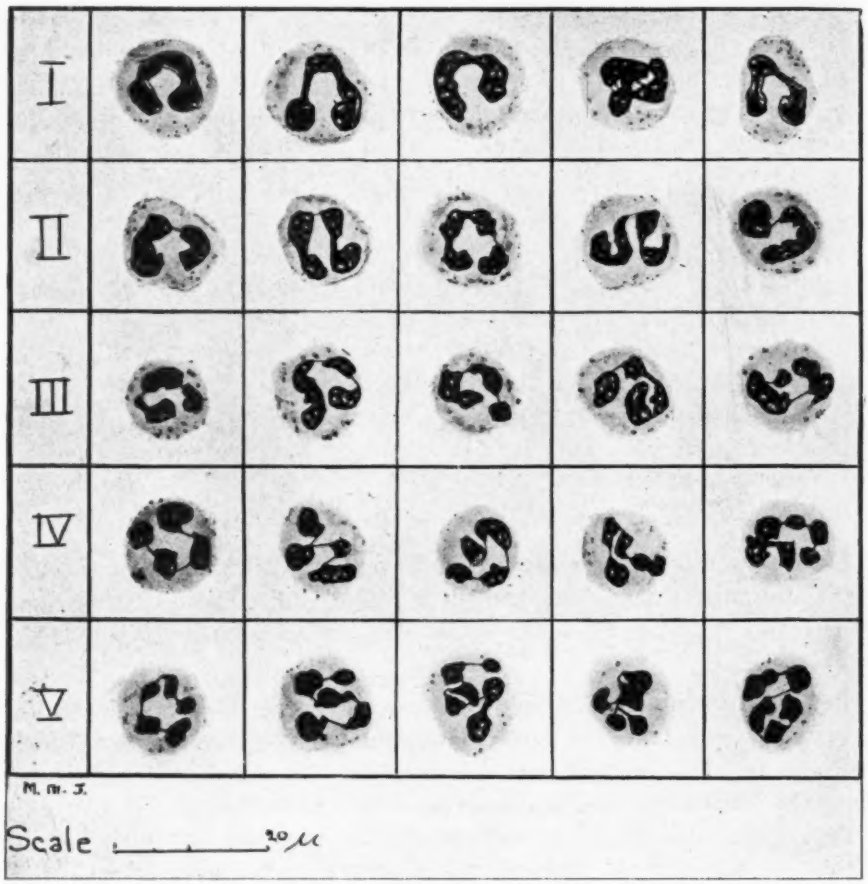

PLATE I.

The five classes of the polynuclear count. If there is any band of nuclear tissue except a chromatin filament, uniting the nuclear masses, those parts must not be considered as separate segments. Class I, No. 2, Class II, Nos. 3 and 4, Class III, No. 4, Class IV, No. 5, illustrate this point. In Class V, No. 4, the upper segment of the nucleus overlies the segment below it, but the thin chromatin filament uniting them can be seen on the left.

Plate II. Fig. 2 is a macropolycyte found in a case of staphylococcal osteomyelitis, which had the following blood count:

Total leucocytes	18,000 per c.mm.
Polymorphs	87 per cent.
Polynuclear count	41, 43, 15, 1, 0.

Plate II. Fig. 3 is a macropolycyte from a case of streptococcal septicemia.

Total leucocytes	26,000 per c.mm.
Polymorphs	79 per cent.
Polynuclear count	34, 43, 21, 2, 0.

Plate II. Figs. 4 and 5 are macropolycytes from a case of mixed infection by Staphylococcus aureus and Bacillus coli.

Total leucocytes	23,700 per c.mm.
Polymorphs	88 per cent.
Polynuclear count	45, 39, 15, 1, 0.

Plate II. Fig. 6 shows a macropolycyte from a case of streptococcal endocarditis.

Total leucocytes	16,000 per c.mm.
Polymorphs	76 per cent.
Polynuclear count	39, 34, 27, 0, 0.

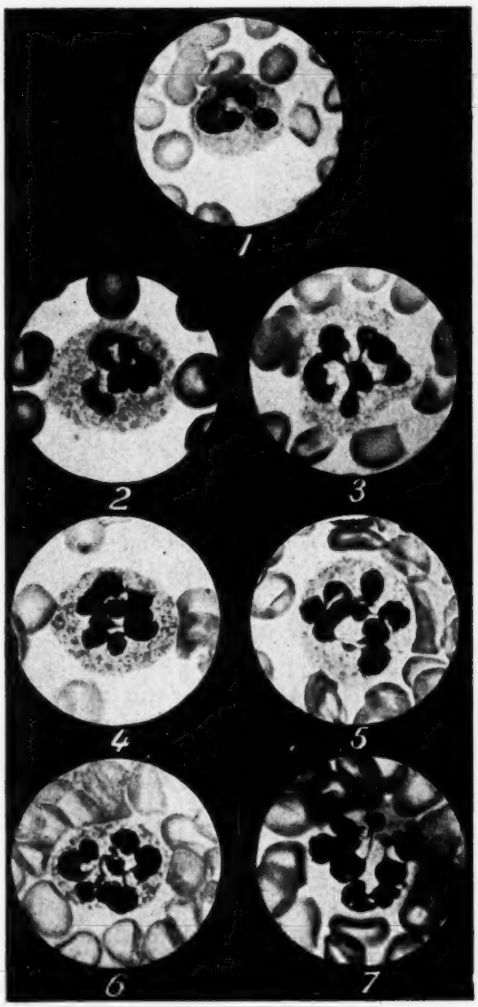

PLATE II.

Fig. 1.—A normal polymorph.

Fig. 2.—Macropolycyte, Type I, from a case of staphylococcal osteomyelitis.

Fig. 3.—Macropolycyte, Type I, from a case of streptococcal septicemia.

Figs. 4 and 5.—Macropolycytes, Type I, from a case of mixed infection by *Staphylococcus aureus* and *Bacillus coli*.

Fig. 6.—Macropolycyte, Type I, from a case of streptococcal endocarditis.

Fig. 7.—Macropolycyte, Type I, from a case of carcinoma of the breast.

(×1,000 diameter.)

Plate II. Fig. 7 is a macropolycyte from a case of carcinoma of the breast.

Total leucocytes	14,500 per c.mm.
Polymorphs	76 per cent.
Polynuclear count	35, 36, 26, 2, 0.

It will be seen that the macropolycytes have hypersegmented nuclei, although polymorphs with single and bilobed nuclei predominate in the blood films, with, in many cases, an occasional myelocyte. What is the explanation of their presence? It is not easy nor particularly important to determine why macropolycytes appear in health and in infections, because they are rare and do not seem to have any prognostic or diagnostic significance. There are two possible explanations of their presence. The first that they represent an occasional polymorph that has escaped the physiologic mechanism whose function is the destruction of aged leucocytes and their removal from the blood stream. A polymorph has been imprisoned in some tissue space, exceeded its allotted span of life, and continued to develop beyond the normal limits. The result is that it has become a giant. The second explanation is that they are cells whose development has been altered by an abnormal medium to which they have been exposed in the marrow during the first period of their development.

THE MACROPOLYCYTES OF PERNICIOUS ANEMIA

1. The Type I macropolycyte is frequently found in pernicious anemia, and here again it is necessary to consider the general behavior of the polymorph in this disease. Small forms, micropolycytes, are often encountered (Fig. 1, Plate III). These cells measure 10 microns or less in diameter, and the nucleus may be single or multilobed. From the micropolycyte all gradations occur up to the macropolycyte. As we have seen above in the infective state the polynuclear count is left-handed. Many uncomplicated cases of pernicious anemia, however, show the remarkable state of having an increase in the cells of Classes IV and V, and the count appears to be actually right-handed, as the following figures show:

	I	II	III	IV	V
Normal	10	25	47	16	2
Case 1	8	20	39	22	11
Case 2	6	27	40	22	5
Case 3	7	20	44	24	5
Case 4	7	16	29	39	9
Case 5	20	31	17	10	10
Case 6	16	22	32	16	14
Case 7	10	15	38	24	13
Case 8	12	34	26	20	8
Case 9	6	20	42	20	12
Case 10	8	24	30	26	12
Case 11	6	18	36	24	16
Case 12	6	20	40	26	8
Case 13	14	28	23	20	15
Case 14	12	28	36	18	6

This condition is not seen in any microbic disease.

It has been pointed out above that no class is made in the polynuclear count for cells with more than five lobes in their nuclei, because cells with more

PLATE III.

Fig. 1.—A polymorph of small size, a micropolycyte.

Figs. 2, 3, 4, 5, 6, 7, and 8.—Polymorphs of normal size showing hypersegmentation of the nucleus.

Figs. 9, 10, 11, 12, 13, and 14.—Macropolycytes, of Type I.

Figs. 15 to 25.—Macropolycytes, of Type II.

(×1,000 diameters.)

than five lobes are rare in normal blood, and if any are found they are placed in Class V. In pernicious anemia, on the contrary, hypersegmentation of the nucleus in cells of normal size is frequent. The nucleus may have six, seven, eight, or even ten or more divisions. Plate III, Figs. 2 to 8 are examples of this

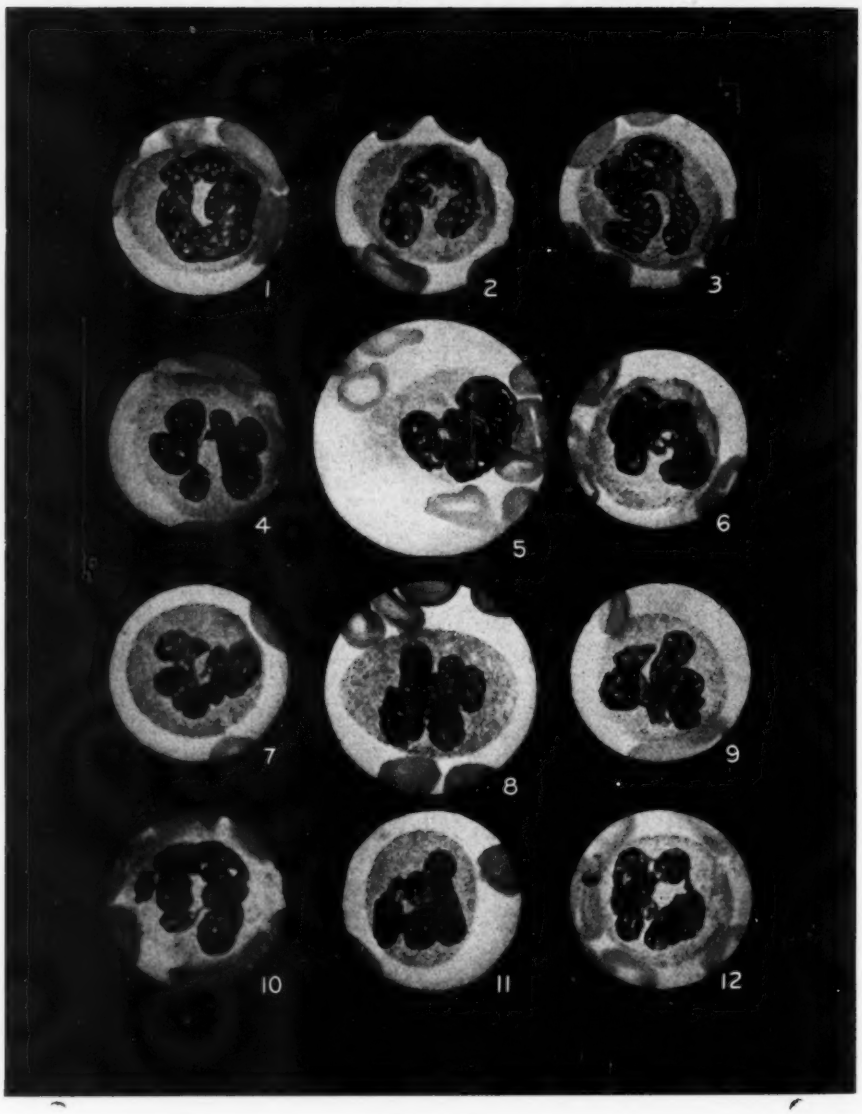

PLATE IV.

Figs. 1 to 12 illustrate the third type of macropolycyte. The figures show the large bulk of the nucleus, the structure of which appears to be a more open meshwork than normal, suggesting deficiency in basichromatin. The fine cytoplasmic oxyphil granules are also seen. (×1,000 diameters.)

nuclear hypersegmentation in cells of approximately normal diameter. Plate III, Figs. 9 to 14 are examples of the Type I macropolycyte. Its frequency in pernicious anemia contrasts very sharply with its rarity in health and in infective conditions, and the cause of its appearance is probably not the same.

There are several possible causative factors. Polymorphs of normal size with hypersegmented nuclei accompany the Type I macropolycyte in this disease, and the first thoughts are that there is some biochemical change in the plasma that prematurely ages the polymorph and alters its life history; the second, that the mechanism for the elimination of old polymorphs is in abeyance. The third reasonable hypothesis may be advanced that the polymorph is inherently abnormal owing to a defect in its parent, the hemocytoblast, or in its environment. Possibly, too there is a single biochemical defect that is responsible for all three conditions. The liver diet, or hog's stomach alters the polymorph picture in a few days. Macropolycytes and polymorphs with hypersegmented nuclei disappear, and at the same time the morphologically and probably also chemically abnormal cells of the hemoglobiniferous series are replaced by cells of normal appearance.

2. The Type II macropolycyte is found only in pernicious anemia, and appears in blood films during serious relapses. Almost all the cases in which it has been found have had erythrocyte counts of less than 1,000,000 per c.mm., and before the era of liver treatment all died within a short time of its appearance. After death these large cells are found in the liver and preaortic glands in association with islets of megaloblasts. Plate III, Figs. 15 to 25, illustrates the variations in this type of cell. The cells are large, measuring up to 24 or more microns in diameter, and resemble the megakaryocyte of the marrow.

3. The third type of macropolycyte, illustrated on Plate IV, is also found in pernicious anemia only. Its characteristics have already been described and are seen in the figures. Like the second type it is found in grave relapses and its appearance before the introduction of modern treatment was of grave prognostic import. We believe this cell is a variant of the preceding type and the explanation of its presence in the blood stream to be the same.

These cells do not resemble any embryonic or postnatal blood stream cell, nor are they accurate replicas of the megakaryocyte of the marrow. The life history would appear similar to that of the polymorph in that they seem to commence circulatory existence with a single-lobed nucleus which later becomes segmented. The length of life we do not know, nor have we any means of calculating it. There is considerable doubt about their origin. They may be an expression of the abnormal reversion to embryonic type of the marrow hemocytoblast as are the hemoglobiniferous cells in pernicious anemia. On the other hand, the cells may not arise in the marrow, but originate in the mesenchyme descendants in the liver and hemolymph glands that have undergone abnormal metaplasia.

CONCLUSIONS

1. The macropolycyte found in health and in infections has no diagnostic nor prognostic significance, and is probably due to the accidental prolongation of the lifetime of a neutrophile polymorphonuclear leucocyte.

2. The causation of the appearance of the Type I macropolycyte in pernicious anemia is closely related with the factor responsible for the production

of hypersegmentation in the nuclei of the polymorph. Whether the factor is a biochemical change in the environment or an inherent defect in its parent cell is not known.

3. The Types II and III macropolycytes may have their origin in the marrow, or may arise from the endothelium of the liver and hemolymph glands. In the first case they are products of abnormal reversion of the hemopoietic tissue, and in the second of abnormal myeloid metaplasia.

Photomicrographs by C. F. Hill and W. E. Cooke.

I most gratefully acknowledge the kind permission of Messrs. Charles Griffin and Co., London, to reproduce Plate I from the Polynuclear Count, Dr. Paul Chevalier, for Plate II from Le Sang, and the Editors of the Journal of the Royal Microscopical Society for Plates III and IV.

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SODIUM RICINOLEATE*†

I. An Attempt to Determine Its Action in the Alimentary Tract

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SODIUM ricinoleate, one of the soaps of castor oil, has been and is at the present time, widely used clinically. It appeared to us that an attempt would be desirable to determine the site of its action and the mechanism of its action when used in the intestinal tract.

Two methods were employed in the attempt to elucidate these points. The first was by means of a dye, Sudan IV or azo-orthotoluene-azo-B.-naphthol. This dye is soluble in oils, ethers, chloroform, fat, fatty acids, and soaps. It is insoluble in aqueous solutions. Sudan IV was first used by Daddi in 1896 in a study of fat absorption. When a fatty acid in which Sudan IV has been dissolved is saponified, the Sudan IV remains with the fatty acid and the result is a pink soap. Also, if the dye is mixed with the soap and hydrochloric acid added, the Sudan IV will remain with the fatty acid radical. In view of this property of Sudan IV, it would seem to be an acceptable guide as to the route taken by the fatty acid radical during digestion. It was found by Mendel and Daniels (1912) that Sudan IV fed alone caused no staining of the fat depots, hence was not absorbed. This was checked by Gage and Fish

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(1924) who found that animals fed boiled rice and cooked egg white with which Sudan IV was intimately mixed showed no absorption which they could detect.

The second method of attack was discovered by Edmunds in 1877 and put to use by S. H. Gage in 1924 in his study on the absorption of fats. In this method the minute fat particles (chylomicrons) in the blood were counted. Edmunds said, "If fresh, transparent blood serum with a few corpuscles shaken out of the edge of the clot be viewed, the serum is seen filled with a nebulous haze of points as is mote laden air in a sunbeam or in the electric light." These small particles were counted by means of a net micrometer in the eyepiece of the microscope and a dark-field illuminator. The particles were easily recognized because of their continuous Brownian movement. The technic used was that of Gage and Fish (1924) in which a small drop of blood was placed on a No. 1 cover slip. This was then put on a slide and the excess blood pressed out. The preparation was oiled around the edge of the cover slip to prevent drying.

With these two methods the action of sodium ricinoleate in the intestinal lumen was studied and compared with that of ordinary fats and fatty acids.

The literature on the subject of fat absorption is not in strict accord. Radziejewski (1868) believed that fats were, for the most part, absorbed as soaps. He based this belief on experiments in which he measured the amount of soap fed to animals and the amount excreted in the feces. He believed there was less in the feces, indicating absorption. Plant (1908) isolated loops of small intestine 55 to 75 cm. in length in which he placed fats and soaps. According to his findings, neutral fats were slightly absorbed and soaps in greater quantity. Such a procedure as Plant's would, presumably, alter physiologic relations considerably. Whitehead (1909) believed that fats were absorbed mostly as soaps with probably a small amount taken up as neutral fat. The work of Clark and Clark (1917) indicated that fat could be taken up by the leucocytes and transported to the lymphatics in the unsplit form. Bloor (1916) said, "The way in which food fat (or at least a part of it) gets from the intestine into the blood has been quite satisfactorily determined. It is saponified in the intestine, absorbed in the water soluble form as soaps and glycerol, resynthesized by the intestinal cells and passed into the chyle and thence to the blood as neutral fat (glycerides) suspended in the plasma in a very finely divided condition." Bang (1918) agreed that fats were absorbed as soaps and were changed back to neutral fats in the intestinal wall. Bloor (1922) still believed that fats could be absorbed as soaps but also said that fatty acids and glycerol could be taken up and resynthesized into fats. In 1925 he stated that fatty acids and glycerol were readily absorbed and that in their passage through the intestinal wall they were resynthesized into fats. There was no statement made about the absorption of soaps.

Verzar and Kuthy (1929) advanced another idea as to the method of fat absorption. They showed that oleic, stearic, and palmitic acids would make clear solutions with sodium glycocholate and sodium taurocholate. This would render fatty acids diffusible and so transportable from the intestinal lumen to the blood stream.

Perhaps the most commonly accepted theory at present is that neutral fats enter the small intestine as such, but at this point the reaction becomes alkaline and the lipase breaks the neutral fat into fatty acids and glycerol. The bile aids by breaking the neutral fat into minute particles so that the lipase will have access to the neutral fat more easily. The fat then enters the intestinal wall as fatty acid and glycerol where it is resynthesized into neutral fat, probably by the lipases found in the intestinal cells. At least the fat is in the neutral state by the time the lacteals are reached.

The question still exists as to the possibility of the absorption of fats as soaps. If fats are absorbed as soaps, a soap made from a fatty acid in which Sudan IV is dissolved to identify the fatty acid radical, in case absorption takes place, indicates its eventual deposition by pink fat depots. Also after giving the soap, the chylomicron (fat particle) count should increase, as it does after a fat meal. According to Bloor, Gillette, and James (1927) the chylomicron count, in general, parallels the quantitative determinations of fat in the blood.

In the studies made in this laboratory on the utilization of fats and soaps in the intestinal tract, white rats were used. Whenever possible the animals were not forcibly fed, but in the case of the soaps this was necessary. Here, under ether anesthesia, a small catheter was passed into the stomach and the soaps introduced. Fearing that ether anesthesia might influence absorption, control animals were anesthetized and fed lard. The chylomicron count showed the same increase as in unanesthetized animals. All animals were starved twenty hours before the experiments. There are shown protocols of animals that were fed lard, butter, oleic acid, castor oil and sodium ricinoleate. Soaps were well tolerated when fed by mouth. Intraperitoneal injections of lard, butter, castor oil and sodium ricinoleate were also made in an attempt to determine if absorption from the peritoneum takes place. The following protocols are illustrative of the results obtained.

PROTOCOLS

Rat 1, on June 4, was given ether anesthesia at 9:30 A.M. as a control on its effect in parallel experiments. It was immediately given a meal of toast and lard in which Sudan IV had been dissolved. Chart 1 shows the chylomicron curve during the digestive cycle. The curve is typical and corresponds to those found by Gage and Fish (1924). June 5 and 6 the animal was given stained lard and was chloroformed June 6. The fat of the inguinal, dorsal, omental and kidney regions was stained pink, suggesting good absorption.

Rat 2 was fed soap made from pure lard containing dissolved Sudan IV. A total of 3.1 gm. of soap was fed in 10 per cent solution from May 11 to May 24. The animal remained in good condition throughout and ate corn and oats freely. May 24 the animal was chloroformed. The fat in the depots and in the omentum was not stained. There was some pink material in the stomach. No evidence of absorption was seen.

Rat 3, May 25 at 11:00 A.M. was fed toast spread with butter in which enough Sudan IV had been dissolved to give the butter a distinct red color. This was eaten freely. May 26 at 11:45 A.M. the animal was again fed colored butter on toast and ate freely. At 3:15 P.M. it was chloroformed. The fat of the mesentery, of the other depots, and around the kidneys was decidedly pink in color which indicated good absorption of the butter.

Rat 4 was fed oleic acid stained with Sudan IV. The chylomicron count was made twenty-four hours later and the results are shown in Chart 2. On each of the two following days the animal was fed 1 c.c. of the stained fatty acid. Four hours following the last feeding the animal was chloroformed. The omental and perirenal fat was stained deeply pink. The fat depots of the groin and back were well stained, and there was some pink fat all through the small intestine. Oleic acid was quite irritating and produced some diarrhea, pink material appearing in the stools. Despite this, there is evidence that oleic acid is absorbed.

Rat 5. Oleic acid containing dissolved Sudan IV was saponified and 1 c.c. of an 18 per cent sodium oleate solution fed daily for four consecutive days. The results of the chylomicron count are shown in Chart 3. On two succeeding days the animal was fed 1 c.c. of the soap and chloroformed four hours after the last feeding. None of the fat depots were stained, although an abundance of pink material remained throughout the lumen of the small intestine. The chylomicron count indicates that there was no fat absorption, as does the absence of stained fat.

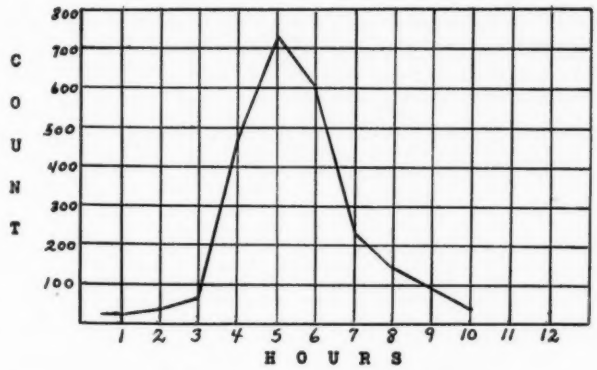

Chart 1.—Rat 1, chylomicron curve.

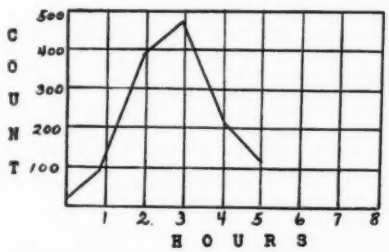

Chart 2.—Rat 4, chylomicron curve.

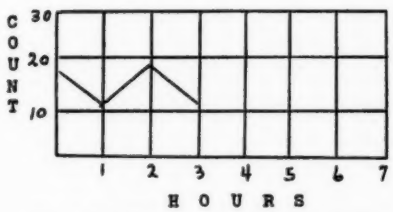

Chart 3.—Rat 5, chylomicron curve.

Rat 6, at 8:00 A.M., was fed toast spread with castor oil in which Sudan IV had been dissolved. Twenty-four hours later it was again fed toast and dyed castor oil and was chloroformed at 1:30 P.M. There was pink material in the stomach and small intestine but none in the colon. No staining of the fat in the mesentery, around the kidneys or in the fat depots in the inguinal or back region was seen, indicating lack of absorption.

Rat 7 was fed 0.23 gm. of sodium ricinoleate in which Sudan IV had been dissolved. The soap was fed as a 10 per cent aqueous solution which formed a clear, transparent pink liquid. The initial dose was given May 9, 1932, and repeated daily until 3 gm. had been fed. May 24 the animal was chloroformed and examined. The fat depots, including the omental fat, were not stained. There was pink material in the stomach and small intestine, but none in the colon. The animal had been given a basic diet of corn and oats and was in good condition at the time of death. There was no evidence of absorption.

Rat 8 was fed 4.3 gm. of sodium ricinoleate in 25 per cent solution from May 21 to May 30, when he died of multiple lung abscesses. There was no staining of the mesentery,

perirenal fat or fat in other depots. The administration of sodium ricinoleate probably had nothing to do with the lung condition.

Rat 9, at 10:00 A.M. was fed 0.2 gm. of sodium ricinoleate by means of a tube, under ether anesthesia. Chart 4 shows the curve of the chylomicron count, the count being plotted on the ordinate and the time on the abscissas. There was no evidence of absorption.

Rat 10 was injected intraperitoneally with 3 c.c. of stained 10 per cent lard soap. Three days later the animal was chloroformed and examined. The perirenal, omental and mesenteric fat appeared pink on the surface, but on cutting there was no penetration of stain into the fat. The fat depot of the dorsal region was unstained. No evidence of absorption was seen.

Rat 11 was injected intraperitoneally with 1 c.c. of butter stained with Sudan IV and the injection repeated the following day. Two days later the animal was chloroformed. There was no staining in any region, including the back, groin, kidney, mesentery, and omentum. The butter and Sudan IV remained in the peritoneal cavity.

Rat 12 was injected intraperitoneally with 1 c.c. of castor oil in which Sudan IV had been dissolved, and this was repeated the following day. Two days later the animal was chloroformed. The material injected still remained in the peritoneal cavity. The omental, perirenal, inguinal and dorsal fat was perfectly white and showed no staining.

Rat 13 was injected intraperitoneally with 3 c.c. of stained 20 per cent sodium ricinoleate and died in three hours. There was no recognizable staining of any body fat. There was

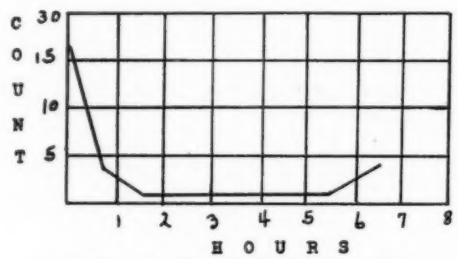

Chart 4.—Rat 9, chylomicron curve.

serous, blood-stained fluid in the peritoneal cavity and the peritoneum was inflamed, but this action is not considered specific for sodium ricinoleate.

There was also noted a very marked increase in the erythrocyte count. We make no explanation of this fact, but did note that 2 c.c. of a 10 per cent silver nitrate solution or 2 c.c. of 2½ per cent nitric acid injected intraperitoneally produced the same results.

Rat 14 at 9:15 A.M., was given 2 c.c. of stained 10 per cent sodium ricinoleate, intraperitoneally. At the time of injection the erythrocyte count was 7,960,000 and at 10:05 the count had risen to 8,200,000. The animal died before another count was made. Examination showed no evidence of fat staining, and there was some fluid in the peritoneal cavity but no more than was injected.

Rat 15, at 10:50 A.M., was injected intraperitoneally with 2 c.c. of stained 5 per cent sodium ricinoleate. Examination showed a small amount of blood in the pleural cavities. There was some soapy material in the peritoneal cavity but no more than was injected, and the peritoneum was inflamed. There was no evidence of staining of the fat in any region. Table I shows the erythrocyte count, white cell count and chylomicron count.

From the above work, based on the study of fat absorption and deposition by the use of Sudan IV and chylomicron counts, it would appear:

1. That the neutral fats, butter, lard, and free oleic acid, are absorbed from the intestinal canal.

2. That the neutral fat, castor oil, is not so absorbed.

3. That soaps made from lard, butter or castor oil (sodium ricinoleate) were not thus absorbed.

4. That none of the above substances were absorbed from the peritoneal cavity.

TABLE I

RAT 15

TIME	ERYTHROCYTE COUNT	WHITE CELL COUNT	CHYLOMICRON COUNT
10:50 A.M.	8,130,000	8,400	90
11:50	9,500,000	5,700	7.5
1:10 P.M.	10,040,000		9
2:20	10,810,000		15
3:00	Animal died		

DISCUSSION AND SUMMARY OF PROTOCOLS

The evidence here presented indicates that soaps are not absorbed from the alimentary tract. This is in direct contradiction to the conclusions of earlier workers who believed that fats are absorbed as soaps, even though a soap splitting enzyme has never been isolated. This work suggests that fats are absorbed as fatty acids and glycerol. We cannot explain the lack of absorption of castor oil. Gage and Fish (1924) also found that it was not digested. They suggested, as a possible explanation, the fact that ricinoleic acid contains more oxygen than do the ordinary fatty acids which are readily absorbed. Perhaps the same explanation may be applied to the soap as to the oil, but, since ordinary fat soaps are not absorbed, this appears to be a question of the soap group rather than of a specific soap.

The results with intraperitoneal injection of lard, butter, sodium ricinoleate and castor oil stained with Sudan IV indicated that these fats and soaps are not absorbed by the peritoneal surface. Castor oil was perfectly innocuous in the peritoneal cavity, in marked contrast to the castor oil soap.

Sodium ricinoleate is apparently not absorbed by the intestinal mucosa, hence any action it may exert should be limited to the lumen of the intestinal canal.

SUMMARY

1. The evidence indicates that oleic acid, butter, and lard are absorbed from the intestinal canal.

2. There is no evidence that soaps or castor oil are absorbed from the intestinal canal.

3. By the methods thus employed, butter, lard, lard soaps, castor oil, and sodium ricinoleate are not absorbed from the peritoneal cavity. Sodium ricinoleate appeared toxic in high concentration.

4. Any action of sodium ricinoleate administered orally would seem to be limited to the intestinal canal. Work is being continued in an effort to identify such action.

5. If the deaths of Rats 13 and 14 and the irritation in Rat 15 are an indication of absorption, we believe it is absorption of a material other than

the fatty acid radical stained with Sudan IV. No attempt to find other methods of absorption was made.

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SODIUM RICINOLEATE*†

II. A Study of the Intestinal Flora During Oral Administration

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SODIUM ricinoleate is used in gastrointestinal disorders which are believed to be due to a disturbance within the tract itself. To what extent these various disturbances are due to intestinal flora or to products of digestion is not known. This paper is a study of the effect of sodium ricinoleate, administered by mouth, on the intestinal flora.

Sodium ricinoleate was probably first used in bacteriologic work by Larson, Cantwell and Hartzell in 1919. They found a solution of sodium ricinoleate very effective in lowering the surface tension of media, by which the growth of bacteria was greatly influenced.

Albus and Holm (1926) reported that when the surface tension of the medium was lowered to forty dynes by means of sodium ricinoleate, the growth of *Lactobacillus bulgaricus* was inhibited, while *Lactobacillus acidophilus* grew well at thirty-six dynes.

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M. Frobisher (1926) found that surface tension is an important factor in bacterial growth. He used sodium oleate as a depressant but not sodium ricinoleate. W. R. Albus (1927), using sodium ricinoleate as a surface tension depressant, found that there was a decrease in the number of living cells in cultures of *E. coli.*

Pizarro (1927) found that inhibition of bacterial growth was not directly associated with surface tension. In many instances the greatest inhibition occurred at higher surface tensions, while broth at lowest surface tension gave the maximum development. This indicates that inhibition is due to the chemical nature of the depressant rather than lowering of surface tension. Day and Gibbs (1928) show that sodium ricinoleate is more toxic to bacteria than other soaps. They found that it is not the depression of the surface tension which affects the bacteria but some other action, possibly chemical. They reported that sodium ricinoleate exerted a more toxic action toward *L. acidophilus* than toward *L. bulgaricus.*

Kozlowski (1928) found that sodium ricinoleate in a dilution of 1:5,000 killed streptococci isolated from cases of erysipelas, measles, and scarlet fever in about seven hours, while control cultures survived for a week or more. Growth was inhibited by a dilution of 1:20,000 or less. Pneumococci were dissolved by a dilution of 1:5,000. *C. diphtheria* was inhibited by a dilution of 1:20,000 and killed by a dilution of 1:2,000 in seven hours. *S. paratyphosus, E. dysenteriae, E. coli,* and *Strep. fecalis* were very resistant to the bactericidal and inhibitory action of sodium ricinoleate. He concluded that specific cellular susceptibility of the bacteria and the concentration of the soap affects its bactericidal and inhibitory action.

Walker (1924) found the staphylococcus had a marked resistance to soaps in general. He found that the laurates had the greatest germicidal action of any soap. Pneumococci and streptococci were the most readily affected. He did not use sodium ricinoleate.

Spencer (1930) found that sodium ricinoleate, in appropriate amounts, completely cleared suspensions of certain species in normal salt solution, notably *B. tularense.* He found that the density of suspensions of some other bacteria was increased by the addition of sodium ricinoleate and in still others the density would be increased at one dilution and decreased at another. He noted that many bacteria would grow in a 1 per cent solution of sodium ricinoleate. Upon observing the action of sodium ricinoleate upon *B. tularense* with the dark-field illuminator, he found that the individual cells lose their refractility as a whole, but retain a few refractile granules for a time, which finally disappear, the resulting solution being noninfectious for guinea pigs.

The literature leaves undetermined the mode of action of sodium ricinoleate. There is no doubt that it has some effect upon bacteria but the mechanism is not clear. Apparently surface tension is not the only factor involved.

Selecting one of the most common members of the intestinal flora, *E. coli,* the percentage of sodium ricinoleate necessary to inhibit growth in cultures was studied. Plain broth was selected as one medium and a filtrate from stools of patients as another. The latter was chosen because conditions as

nearly as possible like those in the intestine were desired. Sodium ricinoleate was added to the media in varying amounts. The tubes were inoculated with a strain of *E. coli* isolated from a human source and incubated at 37° C. for twenty-four hours. Then a loopful was spread on a blood agar plate and incubated for twenty-four hours. Tubes which contained no sodium ricinoleate were used as controls. Tables I, II and III give the results:

TABLE I

SODIUM	TUBES			
Ricinoleate	1	2	3	4
10%	–	–	–	–
9%	–	–	–	–
8%	–	–	–	–
7%	–	–	–	–
6%	–	+	+	+
5%	+	+	+	+
0%	+	+	+	+

Plain broth media

TABLE II

SODIUM	TUBES			
Ricinoleate	1	2	3	4
10%	–	–	–	+
9%	–	–	–	+
8%	–	–	–	+
7%	–	–	–	+
6%	–	–	–	+
5%	–	–	–	+
4%	+	–	+	+
3%	+	–	+	+
2%	+	–	+	+
1%	+	–	+	+
0%	+	–	+	+

Stool filtrate media

TABLE III

SODIUM	TUBES			
Ricinoleate	1	2	3	4
8.3%	–	–	+	+
7.5%	–	–	+	+
6.6%	–	–	+	+
5.8%	–	–	+	+
5.0%	–	–	+	+
4.1%	–	–	+	+
0 %	+	+	+	+

1 c.c. stool filtrate plus 0.2 c.c. broth

Table I demonstrates that at least a 7 per cent solution of sodium ricinoleate is required to completely inhibit the growth of *E. coli* in plain bouillon. In filtrate Tubes 2, Table II, there was no growth in any dilution nor in the controls. In two series of tubes in which nutrient broth was added to filtrate, the growth was inhibited by a 5 per cent solution of sodium ricinoleate while in a fourth series the organisms grew in every dilution. In Table III growth was inhibited in a 4.1 per cent solution in two series of tubes and in two others there was no inhibition in any dilution including the 8.3 per cent sodium ricinoleate. Thus, *E. coli* was quite resistant to the action of sodium ricinoleate, as far as growth is concerned.

The tubes in which there was growth in every dilution were found to have a white precipitate in the bottom resembling a calcium soap. These filtrates were found to contain considerable free calcium by quantitative test. The stools of ten patients who were receiving sodium ricinoleate by mouth were tested qualitatively for calcium, and it was found to be present in all, although in some in small amounts only. This suggests the possibility of sodium ricinoleate being sometimes precipitated as the insoluble calcium salt and rendered inactive when the drug is given by mouth and might indicate the use of much larger therapeutic doses.

In Part I of these papers it was found that the soap is apparently not absorbed from the intestinal tract. It would therefore appear that any action which the drug possesses must be local, and an attempt has been made to ascertain whether any local action can be detected by changes in the intestinal flora. In this, the effect of sodium ricinoleate on the general intestinal flora was studied by direct smear and by cultures. Cultures were made on blood agar plates, in brain broth and in cooked heart medium because each favors a different type of microorganism. No attempt was made to isolate and identify every variety because of the volume of work which would be entailed by such a procedure.

Sodium ricinoleate was administered orally in 5 or 10 gr. gelatin coated capsules. Thirty to 40 grains were given daily to each adult patient. The capsules were given thirty minutes before each meal and at bedtime, as the drug was better tolerated when the stomach was empty.

A specimen of stool was obtained from each patient before sodium ricinoleate was administered and as nearly as possible weekly thereafter. No stool more than five hours old was used. All glassware was autoclaved. Approximately 1 gm. of material from the center of the stool was emulsified with 75 c.c. of sterile isotonic sodium chloride solution. The emulsion was poured into an Erlenmeyer flask, shaken for one and one-half minutes, and centrifuged for thirty seconds at half speed, which was sufficient to throw coarse material down, but left bacteria in suspension. From this suspension, media were inoculated and slides made and stained by Gram's method. The P_H of each stool was determined by the colorimetric technic, using brom-thymol-blue as an indicator. For stools which were more acid, brom-cresol-purple was used. Sodium ricinoleate by mouth apparently did not influence the P_H of the stools. The patients were treated for eight weeks or longer. The direct smears from the stools were stained by Gram's method. Gram stains were made from the brain broth and heart media and from all varieties of colony on blood agar plates after forty-eight hours' incubation at 37° C. This was done prior to beginning the administration of sodium ricinoleate and each week thereafter.

Ten patients were studied in this manner and protocols kept. Table IV is a reproduction of one such protocol. It is considered unnecessary to include the others because, while there were minor variations, the general trends were the same.

TABLE IV

GRAM STAINED SMEARS. MRS. M.

DATE	P_H	STOOL	BLOOD AGAR	BRAIN BROTH	HEART MEDIUM
1932					
3–28	6.8	Coli	Coli Slender Gram+ rods	Coli predominant Few Gram+ rods	Coli Strep. Staph.
4–11	6.8	Coli Gram+ diplococci Large Gram+ rods	Poor growth Staph.	Coli Gram+ diplococci Few large Gram+ rods	Coli Many short coccoid Gram+ rods
4–18	6.6	Coli Gram+ cocci Acidophilus Welchii	Coli Gram+ spore former	Coli, Strep. Gram+ coccoid organisms in chains	Coli Gram+ cocci
4–25	6.4	Coli Gram+ diplococci Acidophilus	Coli	Coli Gram+ cocci Acidophilus	Coli Gram+ diplococci Welchii Gram+ club-shaped rod
5– 2	6.2	Coli Few Gram+ diplococci	Coli	Coli, Strep. Long Gram– rods	Coli Welchii Acidophilus
5–16	6.0	Coli Gram+ diplococci Acidophilus	Coli Small Gram+ rods	Coli Gram+ diplococci Acidophilus	Coli Welchii Gram+ diplococci
5–23	6.5	Coli, Strep. Acidophilus Gram+ diplococci	Coli	Coli Gram+ diplococci Small Gram+ rods in chains	Coli, Welchii Staph. Small Gram+ rods
6– 2	6.5	Coli Short Gram+ rods Large Gram– rods Gram+ cocci	Coli	Coli Gram+ diplococci Strep.	Coli, Welchii Gram+ diplococci Long Gram– rods
6–20	6.5	Coli Gram+ diplococci Welchii Small Gram+ rods Long Gram– rods	Coli	Coli Small Gram+ rods	Coli, Welchii Gram+ diplococci

Patient was given forty grains sodium ricinoleate daily for sixty-four days

SUMMARY

The daily administration of 30 to 40 gr. of sodium ricinoleate by mouth for about eight weeks had no evident effect on the character of intestinal flora as far as was indicated by direct smear and the cultural characteristics observed. All types of bacteria grew as readily during the administration of sodium ricinoleate as before. The ratio of gram-negative to gram-positive organisms was not changed. In these patients every stool seemed to contain 90 per cent or more of gram-negative bacteria, and these were largely colon bacilli. The growth of all organisms in the media employed seemed just as prolific during the administration of sodium ricinoleate as when no medication was given. The P_H of the stools was not appreciably altered by sodium ricinoleate. Larger doses of sodium ricinoleate might be more effective, for some of the drug appears to be precipitated by the calcium in the intestinal

contents. This would be especially true in those cases where the P_H was quite low. That this could be done with impunity is indicated by the fact that doses which were comparatively several times larger were given to white rats for weeks with no ill effects. For instance, one gram was given daily to one rat through a stomach tube, and at no time did it affect its appetite or well-being.

CONCLUSIONS

1. *E. coli* was inhibited by 7 per cent sodium ricinoleate in broth culture.

2. Sodium ricinoleate, in the accepted clinical dosage, does not alter the intestinal flora as far as can be detected by direct smears and cultures in the three media employed.

3. Sodium ricinoleate in like dosage by mouth has no demonstrable effect on the P_H of the stool.

4. The gram-negative gram-positive ratio is apparently not changed by sodium ricinoleate.

5. The precipitation of sodium ricinoleate by calcium in the intestinal canal is worthy of study, as larger doses might be needed to replace that lost in this way.

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THE VARIATIONS OF THE UREA, TOTAL NONPROTEIN NITROGEN, AND CHLORIDE CONCENTRATION IN THE BLOOD FOLLOWING GLUCOSE INGESTION*

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HERRICK[1] and Katayama[2] observed a fall in the chloride content of whole blood following the ingestion of glucose in human subjects. An inverse relationship between the concentration of glucose and chlorides in the blood has also been noted in diabetes mellitus by Gram[3] and McLean,[4] also by Myers.[5] In cases of Bright's disease, Gram[6] has shown that the retention of nonprotein nitrogenous bodies is often accompanied by a reduction in the chloride content and conductivity of the blood. As in diabetes mellitus with high blood sugar, he assumes that the decrease in conductivity (i.e., salts) represents a regulatory process, which tends to stabilize the osmotic pressure of the blood. McLean[6] has observed that there is a progressive diminution in the plasma chlorides in some patients with Bright's disease up to the time of death in uremic coma, and the presumption has been made by others that this decrease may be partly explained as an attempt to maintain an osmotic balance in the face of a rise of the nonprotein nitrogenous substances in the blood. Experiments reported by Ni[7] show that the fall and rise of blood chlorides which occur in pancreatectomized or normal dogs after the injection of insulin are related to the sugar fluctuations, and he assumes such reciprocal changes to be an effort on the part of the body at osmotic and other compensations.

Such compensatory relationships are not uniformly observed during hyperglycemia and nitrogen retention and, as we shall attempt to show later, following the ingestion of glucose in normal and diabetic subjects. Mosenthal[8] has recently demonstrated that the plasma cholesterol following glucose ingestion may either rise or fall markedly as the blood sugar increases, and consequently the assumption that a reciprocal variation of these two substances maintains the osmotic equilibrium of the blood does not suffice as an explanation for all changes. Experiments carried out in this laboratory on the variations of the plasma proteins during induced hyperglycemia (to be published later) show that the protein fractions of the blood may either rise or fall considerably under such circumstances. It is conceivable when reciprocal relationships between certain constituents in the blood were not observed, that other substances, the

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concentrations of which were not investigated, may have varied in such a way as to maintain a constant osmotic pressure of the blood.

MATERIAL AND METHODS

Sixty glucose tolerance tests were carried out in 54 unselected subjects. The variations of the sugar, urea nitrogen, total nonprotein nitrogen and chloride concentrations in the blood were followed at intervals, in most cases, of twenty minutes, forty minutes, one hour, two hours, and three hours following the ingestion of 100 gm. of glucose. The test was carried out in every instance in the morning, the subject having taken no food since the evening preceding the test. The volume output and the sugar content of the urine were also noted. The blood sugar was determined in every case and the patient's tolerance for ingested carbohydrate interpreted according to the standards proposed by Mosenthal.[9] The variations of the urea nitrogen concentration in the blood were followed in 45 cases, the total nonprotein nitrogen in 13, the whole blood chlorides in 17 and the plasma chlorides in 6.

The methods employed were as follows: urea nitrogen, gasometric urease method of Van Slyke;[10] total nonprotein nitrogen, Folin and Wu;[11] sugar, Folin and Wu;[12] whole blood and plasma chlorides, Short and Gellis.[13]

RESULTS

Urea Nitrogen.—Table I illustrates the variations of the urea nitrogen content of the blood in 45 subjects following glucose ingestion. Assuming deviations of ± 1.0 mg. per cent or more of urea nitrogen as significant, the following findings are demonstrated: *Progressive Fall:* (22 instances) Cases 5 to 8, 10, 13, 15, 17, 19, 22, 24, 25, 27, 30, 32 to 34, 37, 39, 41, 43, and 44. *Preliminary Rise and Later Fall:* (12 instances) Cases 2, 4, 11, 16, 20, 26, 28, 31, 35, 38, 40, and 42. *No Significant Change:* (6 instances) Cases 1, 9, 12, 21, 29, and 36. *Progressive Rise:* (4 instances) Cases 14, 18, 23, and 45. *Preliminary Fall and Later Rise:* (1 instance) Case 3.

Apparently, there is no constant reciprocal relationship between the urea and sugar content of the blood. Cases 5 to 8 and 10 show a persistent diminution in the urea nitrogen while the blood sugar has returned to the control level (normal sugar curves). Again, in Cases 31, 35, 38, 40, and 42 an initial rise and later fall in the urea nitrogen are associated with a persistently elevated blood sugar (high prolonged sugar curves).

A marked fall in the urea nitrogen content of the blood (3 mg. per cent or more) is observed in only one out of eleven subjects (9 per cent) with normal carbohydrate tolerance (Case 5). On the other hand, in the 25 subjects with definitely diminished sugar tolerance (high prolonged sugar curves), we record six (24 per cent) such instances (Cases 24, 27, 32, 37, 43, and 44). Two cases in each group show an increment of 3 mg. per cent or more in the urea nitrogen (Cases 3, 4 and 40, 45, respectively).

Our results demonstrate that following the ingestion of glucose the urea nitrogen content of the blood may rise or (and) fall or show no marked change. A diminution of 1 to 7 mg. per cent, however, occurs in about 50 per cent of the cases, and evidence is presented that an appreciable fall (3 mg. per cent or

TABLE I

THE EFFECT OF GLUCOSE INGESTION ON THE UREA NITROGEN CONTENT OF THE BLOOD

CASE	UREA NITROGEN (MG. PER 100 C.C.)								INTERPRETATION OF GLUCOSE TOLERANCE TEST	URINE VOLUME OUTPUT* C.C. PER MIN.
	CONTROL	20 MIN.	40 MIN.	1 HR.	2 HR.	3 HR.	MAXIMAL INCREASE ABOVE CONTROL	MAXIMAL DECREASE BELOW CONTROL		
1	9.1	9.1		9.4	8.7	8.7	0.3	0.4	Normal	1.6
2	13.7	14.7		13.7	13.7		1.0	--	Normal	0.5
3	8.9	6.8	6.8	8.9	12.3		3.4	2.1	Normal	1.2
4	16.0	20.2		16.0	17.0		4.2	—	Normal	0.7
5	20.9	20.8	14.3	18.6	14.9		—	6.6	Normal	2.3
6	13.0	11.2	11.2	10.5	10.8	10.1	—	2.9	Normal	3.6
7	7.1	7.3	7.1	7.5		5.0	0.4	2.1	Normal	2.4
8	15.4	15.3	14.2	15.2	14.9	13.9	—	1.5	Normal	6.2
9	13.1	13.6	13.6	13.6	12.2		0.5	0.9	Normal (Renal glycosuria)	0.7
10	15.3	15.2	14.6	14.9	13.4		—	1.9	Normal (Renal glycosuria)	1.6
11	13.6	15.6	15.1	13.7	11.7		2.0	1.9	Normal (Renal glycosuria)	5.5
12	10.6			9.9	10.1		—	0.7	Low	1.4
13	13.8	12.6	12.8	12.6	12.0		—	1.8	Low	0.8
14	15.4	17.1	16.3	17.3	17.0		1.9	—	Low	2.7
15	13.1	12.3		11.5	11.0		—	2.1	High	0.8
16	11.6	12.9	14.1	13.8	10.8		2.5	0.8	High	0.9
17	12.7	12.2	11.6	11.9	10.4	10.9	—	2.3	High (Renal glycosuria)	2.0
18	9.0	10.1	9.6	9.0	10.1		1.1	—	Prolonged	0.7
19	22.0	19.6		21.4	19.7		—	2.4	Prolonged	1.0
20	16.1	20.7	15.0	15.2			4.6	1.1	Prolonged	1.5
21	8.2			8.7	9.1		0.9	—	High prolonged	1.2
22	9.7	10.0	9.8	9.4	8.7	7.6	0.3	2.1	High prolonged	0.6
23	10.9	12.1	11.2	11.0	11.5		1.2	—	High prolonged	1.1
24	24.2	21.9	22.0	19.9	22.1		—	4.3	High prolonged	2.1
25	11.1	12.0	11.2	9.9	9.6	9.7	0.9	1.5	High prolonged	2.6
26	6.9	5.7	5.9	8.3	7.2	5.4	1.4	1.5	High prolonged	4.2
27	16.3	15.0	15.4	15.0	13.4		—	2.9	High prolonged	4.1
28	11.5	11.9	11.4	12.8	10.7	11.4	1.3	0.8	High prolonged	0.6
29		15.1		14.7	15.1				High prolonged	0.8
30	22.0	19.6		21.4	19.7		—	2.4	High prolonged	1.0
31	13.2	14.1	14.7	13.8			1.5	—	High prolonged	1.3
32	16.7	14.4	11.6	12.5	10.7	9.6	—	7.1	High prolonged	0.5
33	16.5	13.7	15.3		15.8		—	2.8	High prolonged	0.5
34	6.5	5.2					—	1.3	High prolonged	0.9
35	15.1	16.4	16.9	16.7	15.5		1.8	—	High prolonged	0.6
36	17.1	17.6	16.5	17.0	17.0		0.5	0.6	High prolonged	1.4
37	14.5			11.4	12.0		—	3.1	High prolonged	1.0
38	11.8	12.1		14.4	11.7		2.6	0.1	High prolonged	1.9
39	15.1	14.8	13.7	13.6	12.2		—	2.9	High prolonged	2.1
40	10.0			13.4	10.4		3.4	—	High prolonged	0.9
41	12.3	11.0		11.4	11.0		—	1.3	High prolonged	0.8
42	20.9	21.0	23.1	20.9	21.2		2.2	—	High prolonged	2.2
43	18.8	19.8		12.5	12.5		1.0	6.3	High prolonged (Renal glycosuria)	1.2
44	16.2	14.2	13.7	14.8	12.9		—	3.3	High prolonged (Renal glycosuria)	2.1
45	11.9	13.4	12.4	11.9	16.2		4.3	—	High prolonged (Renal glycosuria)	3.5

*Calculated from the total urine volume output following the glucose ingestion until the termination of the test.

more) occurs more often in subjects with diminished carbohydrate tolerance than in those exhibiting normal tolerance for sugar.

TABLE II

THE EFFECT OF GLUCOSE INGESTION ON THE NONPROTEIN NITROGENOUS CONSTITUENTS OF THE BLOOD

CASE	NONPROTEIN NITROGEN (MG. PER 100 C.C.)								UREA NITROGEN (MG. PER 100 C.C.)		N.P.N.—UREA N (URIC ACID, CREATININE, AMINO ACID AND REST N)								INTERPRETATION OF GLUCOSE TOLERANCE TEST	URINE VOLUME OUTPUT* C.C. PER MIN.
	CONTROL	20 MIN.	40 MIN.	1 HR.	2 HR.	3 HR.	MAXIMAL INCREASE ABOVE CONTROL	MAXIMAL DECREASE BELOW CONTROL	MAXIMAL INCREASE ABOVE CONTROL	MAXIMAL DECREASE BELOW CONTROL	CONTROL	20 MIN.	40 MIN.	1 HR.	2 HR.	3 HR.	MAXIMAL INCREASE ABOVE CONTROL	MAXIMAL DECREASE BELOW CONTROL		
1	29.5	27.6		26.9	29.3	26.9	—	2.6	0.3	0.4	20.4	18.5		17.5	20.6	18.2	0.2	2.9	Normal	1.6
6	31.6	33.0	31.9	31.1	29.1	29.0	1.4	2.6	—	2.9	18.6	21.8	20.7	20.6	18.3	18.9	3.2	0.3	Normal	3.6
7	30.0	29.3	33.2			24.5	3.2	5.5	0.4	2.1	22.9	22.0	26.1			19.5	3.2	3.4	Normal	2.4
8	35.6	34.2	32.7	33.0	31.6	30.7	—	4.9	—	1.5	20.2	18.9	18.5	17.8	16.7	16.8	—	3.5	Normal	6.2
12	31.4			35.1	27.3		3.7	4.1	—	0.7	20.8			25.2	17.2		4.4	3.6	Low	1.4
15	30.8	32.4		33.0	33.9		3.1	—	—	2.1	17.7	20.1		21.5	22.9		5.2	—	High	0.8
21	27.7			29.3	26.7		1.6	1.0	0.9	—	19.5			20.6	17.6		1.1	1.9	High prolonged	1.2
22	30.3	30.5	33.3	26.8	28.2	30.0	3.0	3.5	0.3	2.1	20.6	20.5	23.5	17.4	19.5	22.4	2.9	3.2	High prolonged	0.6
26	20.6	20.1	22.2	25.0	19.8	19.7	4.4	0.9	1.4	1.5	13.7	14.4	16.3	16.7	12.6	14.3	3.0	1.1	High prolonged	4.2
28	35.3	28.0	35.5		30.9	26.0	0.2	9.3	1.3	0.8	23.8	16.1	24.1		20.2	14.6	0.3	9.2	High prolonged	0.6
29	31.3	37.7		37.7	39.2		7.9	—	—	—		22.6		23.0	24.1				High prolonged	0.8
30	38.5	36.4		36.5	35.6		—	2.9	—	2.4	16.5	16.8		15.1	15.9		0.3	1.4	High prolonged	1.0
37	35.5			33.3	30.8		—	4.7	—	3.1	21.0			21.9	18.8		0.9	2.2	High prolonged	1.0

*Calculated from the total urine volume output following the glucose ingestion until the termination of the test.

Total Nonprotein Nitrogen.—Table II illustrates the variations of the total nonprotein nitrogen content of the blood and the sum total of the nonprotein nitrogenous substances excluding urea in thirteen subjects following glucose ingestion. The following findings are demonstrated:

1. The total nonprotein nitrogen may rise or (and) fall or remain unchanged.

2. The total nonprotein nitrogen shows a progressive fall in many instances and is paralleled by the variations of the urea nitrogen in Cases 6, 22, 26, 30, and 37. In these instances, the diminution in the total nonprotein nitrogen is due mainly to the decrease in the urea nitrogen fraction.

3. The sum total of uric acid, creatinine, amino acid, and rest nitrogen may either rise or (and) fall or show no marked change.

Whole Blood and Plasma Chlorides.—Table III shows the variations of the whole blood and plasma chlorides in seventeen and six cases respectively following the ingestion of glucose. In the six instances with plasma chloride studies, simultaneous determinations of the whole blood chlorides were made. The following findings are demonstrated:

1. A reciprocal relationship between the whole blood chlorides and sugar in Cases 46, 48, 50, 32, 52, 53, 55, and 56. In the first three cases, the fall and subsequent rise in the chlorides are associated with an increase of the blood sugar and a later return to the control level (normal and high sugar curves). In the latter five instances, the persistent diminution in the chloride accompanies a prolonged elevation of the blood sugar (high prolonged sugar curves).

2. Nine subjects show either no change or an augmentation of the whole blood chlorides.

3. The variations of the plasma chlorides parallel the whole blood chlorides in Cases 52, 55, and 56. In Cases 47, 48, and 49, the changes in the blood chlorides are due mainly to variations of the chloride content of the plasma.

DISCUSSION

In 1916, Hiller and Mosenthal[14] showed that the ingestion of 100 gm. of glucose by normal individuals was not accompanied by hydremic plethora. We carried out hematocrit studies in a few instances, and we were able to satisfy ourselves that little if any blood dilution occurred. In fact, we observed a slight degree of blood concentration following glucose administration in some cases. The variations of the constituents investigated, therefore, cannot be explained by changes which develop in the water content of the blood caused by the ingestion of 100 gm. of glucose dissolved in approximately 250 c.c. of water.

If the variations of the urea and other nonprotein nitrogen fractions of the blood and of chloride, plasma proteins, cholesterol, etc., following glucose ingestion represent attempts on the part of the organism to maintain a normal osmotic pressure of the blood, then it becomes clear why one constituent (e.g., urea) may rise, fall, or remain unchanged while another substance or substances (e.g., chloride, cholesterol, etc.) may vary in such a way that the summation of their individual osmotic effects is constant. It is conceivable that the regulation is a property residing in all the body tissues and maintained by an interplay of the electrolytes and nonelectrolytes in tissue fluid and blood.

TABLE III

THE EFFECT OF GLUCOSE INGESTION ON THE CHLORIDE CONTENT OF WHOLE BLOOD AND OF PLASMA

CASE	WHOLE BLOOD CHLORIDES (MG. PER 100 C.C.)								PLASMA CHLORIDES (MG. PER 100 C.C.)								INTERPRETATION OF GLUCOSE TOLERANCE TEST	URINE VOLUME OUTPUT* C.C. PER MIN.
	CONTROL	20 MIN.	40 MIN.	1 HR.	2 HR.	3 HR.	MAXIMAL INCREASE ABOVE CONTROL	MAXIMAL DECREASE BELOW CONTROL	CONTROL	20 MIN.	40 MIN.	1 HR.	2 HR.	3 HR.	MAXIMAL INCREASE ABOVE CONTROL	MAXIMAL DECREASE BELOW CONTROL		
46	464	406	473	453	464		9	58									Normal	7.9
12	419			483	419		64	—									Low	1.4
15	459	465		457	455		6	4									High	0.8
47	515	525	515	515	515		10	—	635	575	595	575	635		—	60	High	0.3
48	545	545	535	545	545		—	10	615	635	635	635			20	—	High	5.4
49	485	495	495	495	495		10	—	615	685	625	695	695		80	—	High	2.3
50	495	455	435	451	463	463	—	60									High	4.2
21	445			443	441		—	4									High prolonged	1.2
22	423	427	440	375	415	439	17	48									High prolonged	0.6
26	491	483	511	499	481	485	20	10									High prolonged	4.2
32	465	455	445	465	445	455	—	20									High prolonged	0.5
51	513			513	513		—	—									High prolonged	3.4
52	475	465		481	471	451	6	24	547	555		525	535	517	8	30	High prolonged	0.6
53	540	527		486	473	473	—	67									High prolonged	1.7
54		462	443	462	495												High prolonged	3.6
55	537	543	530	532	515		6	22	615	625	615	635	605		20	10	High prolonged	2.4
56	535	495	535	495	495		—	40	655	635	595	615	635		—	60	High prolonged	2.0

*Calculated from the total urine volume output following the glucose ingestion until the termination of the test.

No apparent relationship was observed between the degree of diuretic response to glucose and the variations of the several constituents in the blood. Urea, as an nonthreshold body, is capable of a "washing-out" process, but Mosenthal and one of us[15] have shown that a marked degree of diuresis in human subjects does not influence materially the amount of urea cleared by the kidney in a unit of time. Obviously, the large amount of urea in tissues and blood is not affected appreciably by the increased rate of urine excretion. The interesting observation was made that diuresis following glucose administration occurred more often and was more marked in subjects exhibiting normal sugar tolerance than in those with diminished tolerance for carbohydrate. As a rule, mild and severe diabetics are dehydrated to a greater or lesser extent and for this reason it is probable that glucose ingestion in these cases is less apt to produce diuresis.

SUMMARY

The ingestion of 100 gm. of glucose by fasting subjects produced the following effects: (A) The urea nitrogen content of the blood may rise or (and) fall or remain unchanged. A gradual fall in the urea nitrogen (1 to 7 mg. per cent) was observed in 22 out of 45 cases. A definite decrease (3 mg. per cent or more) occurred more often in subjects with diminished carbohydrate tolerance than in those with normal tolerance. (B) The total nonprotein nitrogen of the blood may rise or (and) fall or show no marked change. In many cases, though not in all, the variations in the total nonprotein nitrogen were due mainly to changes in the urea nitrogen fraction. Irregular changes were noted in some instances in the sum total of the nonprotein nitrogenous substances in the blood other than urea. (C) There was a distinct reciprocal relationship between the whole blood and plasma chlorides and the glucose concentration in the blood in many instances, though this was not observed in all the cases studied.

There was no apparent relationship between the degree of diuretic response to glucose and the variations in the constituents investigated. Diuresis following glucose ingestion occurred more frequently and was more marked in subjects with normal carbohydrate tolerance than in those exhibiting a diminished tolerance for sugar.

The hypothesis is suggested that these fluctuations represent an effort by the body to maintain a constant osmotic pressure of the blood. The compensatory response of urea, total nonprotein nitrogen, and chloride to a rise of glucose in the blood is not a uniform reciprocal relation. The varied increase or decrease of these substances suggest that the total osmotic pressure is adjusted according to the physiologic reserve prevailing in the body at a given moment.

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THE PERENNIAL TREATMENT OF HAY FEVER*

A Comparative Study

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In 1911, Noon[1] and Freeman[2] introduced the method of preseasonal desensitization of hay fever with specific pollen extracts. Their technic consisted of the subcutaneous inoculation into a sensitive patient of a number of increasingly potent pollen doses during the course of from ten to fifteen weeks preceding the date of expected pollination. While the merits of this treatment have since been generally recognized, the efficacy of their technic has been marred by the following objections: the inconvenience caused to the patient by the necessity of frequent visits to the office or clinic throughout the treatment period; the occasional need of a longer treatment period than allotted, in refractory cases; and finally, the rapid loss of the patient's acquired tolerance for pollens in the interim between treatment seasons.

In 1926 Zella W. Stewart[3] suggested the "throughout the year treatment of hay fever," which, she claimed, met the above-mentioned objections effectively. By this method the patient is reinjected with an optimal dose of pollen extract at intervals of from two to four weeks throughout the year, thus maintaining his acquired immunity. In 1927 and again in 1932 Aaron Brown,[4] working independently, described the "perennial" treatment of hay fever, for which he claimed similarly favorable results.

Additional observations on the perennial method of pollen therapy have since been made by Vander Veer, Cooke and Spain,[5] Figley,[6] Thommen,[7] Vaughan,[8] and Unger.[9] In their final analysis these authors subscribed to all or most of the following advantages of the perennial over the preseasonal

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method of hay fever treatment: fewer office visits, better clinical end-results, greater convenience to the patient, and the possibility of permanent cures. They varied, however, on the incidence of constitutional reactions, which occurred more frequently by the perennial method in the series of Vander Veer, Cooke and Spain, and Thommen, as against a lesser incidence of similar reactions in the series of Figley, Vaughan, and Unger.

The magnitude of the problem of hay fever and the desire to elucidate further the merits of this innovation in its treatment prompted the undertaking of the study set forth in this communication.

Altogether 52 patients were treated by the perennial method during the year ending October, 1932. Of these, 34 patients had formerly received preseasonal treatment for one or more years and form the basis for comparing these two methods on the same group of patients in different years. At the same time 46 other patients who received preseasonal treatment in 1932 serve as additional material for comparing the perennial and preseasonal methods of treatment during the same season, though on different patients.

In Tables I, II, III, and IV, the results of our observations are tabulated under corresponding headings. It is evident that "complete relief" was about

TABLE I

A COMPARISON OF THE END-RESULTS IN 34 HAY FEVER PATIENTS TREATED PERENNIALLY IN 1932 AND PRESEASONALLY IN 1931

	1932 PERENNIAL	1931 PRESEASONAL
Complete relief	24 per cent	12 per cent
Marked relief	67 per cent	76 per cent
Slight relief	6 per cent	12 per cent
No relief	3 per cent	----------
Total per cent of good results	91 per cent	88 per cent

TABLE II

A COMPARISON OF THE AVERAGE NUMBER OF DOSES, AVERAGE AMOUNT OF POLLEN IN 1-100 DILUTION AND OF THE INCIDENCE OF CONSTITUTIONAL REACTIONS IN 34 HAY FEVER PATIENTS TREATED PERENNIALLY IN 1932 AND PRESEASONALLY IN 1931

	1932 PERENNIAL	1931 PRESEASONAL
Average number of doses	22	19
Average amount of pollen in 1-100 dilution	5.60 c.c.	2.04 c.c.
Constitutional reactions	0.06 per cent (1 reaction in 152 treatments)	0.07 per cent (1 reaction in 131 treatments)

TABLE III

A COMPARISON OF THE END-RESULTS OF HAY FEVER TREATMENT BETWEEN 52 PERENNIAL AND 45 PRESEASONAL CASES IN 1932

	PERENNIAL	PRESEASONAL
Complete relief	21 per cent	13 per cent
Marked relief	73 per cent	71 per cent
Slight relief	4 per cent	13 per cent
No relief	2 per cent	3 per cent
Total per cent of good results	94 per cent	84 per cent

TABLE IV

A COMPARISON OF THE AVERAGE NUMBER OF DOSES, AVERAGE AMOUNT OF POLLEN IN 1-100 DILUTION AND OF THE INCIDENCE OF CONSTITUTIONAL REACTIONS IN 52 PERENNIAL AND 45 PRESEASONAL CASES IN 1932

	PERENNIAL	PRESEASONAL
Average number of doses	24	21
Average amount of pollen in 1-100 dilution	5.89 c.c.	2.62 c.c.
Constitutional reactions	0.06 per cent (1 reaction in 156 treatments)	0.04 per cent (1 reaction in 236 treatments)

twice as frequent with the perennial method as compared with the preseasonal in both groups, while the instances of "slight relief" and "no relief" were more common in the preseasonal group. The amount of "marked relief," however, which the bulk of the patients experienced, was not appreciably different in the two groups. Altogether the group of 34 patients who were treated preseasonally in 1931 and perennially in 1932 obtained 88 and 91 per cent of good results in the respective years, while the other two groups, one of 45 preseasonal cases and the other of 52 perennial cases treated in 1932 experienced 84 and 94 per cent of good results respectively. For the slightly better total good results in the perennially treated cases, each patient received an average of three more doses and a total of about twice as much pollen as the preseasonally treated patients.

As seen from our tables, constitutional reactions occurred with slightly greater frequency in the 52 patients treated perennially in 1932 and in the 34 patients treated preseasonally in 1931. It is well to mention here that altogether we had 3 constitutional reactions which required adrenalin. Two of these occurred in perennially treated patients and one in a preseasonal patient. All other reactions were mild and needed no special medication.

TABLE V

ELEVEN CASES WHO OBTAINED BETTER RELIEF IN 1932 BY THE PERENNIAL METHOD

	1931 PRESEASONAL	1932 PERENNIAL
11 Cases	4 patients, slight relief	Marked relief
	7 patients, marked relief	Complete relief

FOUR CASES WHO OBTAINED WORSE RESULTS IN 1932 BY THE PERENNIAL METHOD

	1931 PRESEASONAL	1932 PERENNIAL
4 Cases	1 patient, complete relief	No relief
	2 patients, complete relief	Marked relief
	1 patient, marked relief	Slight relief

Of considerable interest is a group of 11 patients who obtained better relief in 1932 when treated by the perennial method than in 1931 when treated preseasonally. Against these we have 4 patients whose results in 1932 were inferior to those in 1931.

We were unable to standardize the time interval between treatments in the perennial group due to variation in changes in the individual patient's tolerance for pollen as determined by repeated serial skin tests and by the

extent of the local reactions from the previous dose. Most patients were treated at three-week intervals, some at four-week intervals, while a few patients could not maintain their tolerance any longer than two weeks. Two patients who had been refractory to treatment in the past received inoculations practically every week with excellent therapeutic results. In addition all patients were given weekly treatments throughout the pollinating season.

COMMENT

It is evident from this study that the perennial method of pollen therapy which is gaining considerable impetus in its clinical application is not infallible. While it offers a somewhat higher total of "good results" from treatment, it proved inadequate in about 12 per cent of our patients who had better relief when treated by the preseasonal method. Furthermore, in our series, the perennial method necessitated a larger number of visits per patient, and it showed no diminution in the incidence of constitutional reactions as compared with the preseasonal method. The one factor that seems to be decidedly in favor of the perennial method is the time element which permits of sufficient treatment in the refractory type of patient as evidenced by two of our cases who required inoculations at weekly intervals, throughout the year.

Of additional interest is the patient's personal reaction to this newer method of treatment. Those who could tolerate treatment at four-week intervals did not mind the occasional visits, while others who had to come in once every two weeks, resented the thought of the "endless visits to the doctor." They felt that eight or even seven months of freedom from treatment was a desirable period for relaxation.

Particularly unpleasant were those few instances when patients, though satisfied with the preseasonal method, acceded on our advice to the more trying perennial method, only to find the end-results less satisfactory. A case in point is the following: F. V. had complete relief in 1929, 1930, and 1931 by the preseasonal method of treatment, while in 1932, by the perennial method, his relief was only partial, although his maximum dose was the same each year. It is apparent that the frequency of administration of pollen doses may in certain instances influence the end-results in therapy.

As to the possibility of permanent cures by the perennial method, we cannot voice any opinion, since this study is based on the experience of one year's treatment. Should the permanent results ultimately prove to be better, one may disregard the several disadvantages incidental to the perennial method of treatment and advocate it with fewer reservations. For the present, however, we must accept this method only as "another method" applicable in selected cases, the most important indication for its use being refractoriness to treatment, where the time element is concerned.

Sight must not be lost of those instances pointed out by Rackemann,[10] whose treatment by the preseasonal method may be reduced to six or even three doses a year, which is an impossibility by the perennial method in the best of hands.

Finally, a great many pollen sensitive patients are treated by the practitioner who may find the perennial method more complicated and less safe to the patient than the more simple preseasonal method of desensitization.

SUMMARY

A comparative study of the perennial and preseasonal methods of hay fever therapy indicate that:

1. The total good results from treatment were somewhat higher by the perennial method.

2. In about 12 per cent of the cases the results obtained by the perennial method were inferior to those produced by the preseasonal method of treatment.

3. The number of treatments per patient were greater by the perennial method.

4. Constitutional reactions occurred with slightly greater frequency in the perennially treated group.

5. The technic of treatment is far more complicated by the perennial method, while the convenience to the patient seems greater when treated by the preseasonal method.

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REFLEX EOSINOPHILIA*

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SEVERAL years ago one of us† produced emphysema with accompanying asthmatic syndrome in dogs by means of an intratracheal ball valve. Briefly, this condition was induced by the introduction of a ball valve into the trachea which would partially obstruct expiration without, in any way, affecting inspiration. Fig. 1 shows the valve in position in the trachea. This experiment led the authors to believe that a possible relationship exists between eosinophilia and mechanical expiratory dyspnea.

Previously, Freedman and Jackson[1] produced temporary expiratory obstruction in dogs for the purpose of studying the carbon dioxide values of the blood and of alveolar air. Their obstruction was brought about by employing a one-way valve attached to a tracheal T-tube. The results were obtained during experiments conducted under anesthesia.

Later, Pescatori[2] produced respiratory insufficiency by blocking the trachea with oil or tap water. Under these conditions he reported an eosinophilia which ran from 5 to 25 per cent. Unfortunately, it was impossible by this local edema method to maintain a constant tracheal pressure. Neither did this method permit regulation of the expiratory phase of respiration without affecting normal inspiration, because tracheal edema per se will alter both phases of respiration. Pescatori believes that the chronic stimulation of the tissues resulting from pathologic conditions, such as pneumothorax, pleurisy, etc., is of an acidotic nature and that this is the cause of the accompanying eosinophilia. To support this theory he poisoned rabbits rapidly with carbon dioxide (asphyxia) and obtained a high grade eosinophilia.

In our experiments blood studies were made before inserting the valve in the trachea of dogs to ascertain normal eosinophile values, and daily thereafter, during intubation. Further studies were carried during the acute asthma attacks and lastly at intervals following the spasms. The results of these studies are tabulated in Table I.

Our observation periods for dogs varied from twenty-four hours to seven days. During these periods aside from the expiratory dyspnea (asthma attack) which was purposely elicited (by exercise or the inhalation of tobacco smoke) for blood sampling, the general health of the animals was excellent.

In this table data are presented showing that, following the retardation of the expiratory phase of respiration, the eosinophile count increased on an

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†For complete detail consult "The Experimental Production in Dogs of Emphysema with Associated Asthmatic Syndrome by means of an Intra-Tracheal Ball Valve." J. Exper. Med. 30: 75, 1919.

average of 4 per cent, which was upon occasion increased by exercise or inhalation of smoke as high as 18 per cent. The type of respiration thus brought about produced in the animals every clinical point required for the diagnosis of asthma; and in addition, continued expiratory difficulty resulted in permanent emphysema.

In each case the total white cell count increased shortly after intubation, in two cases going as high as 15,000. This leucocytosis corresponds with that found in human beings during exercise. The eosinophile count increased to an average of 4 per cent on the first day, and to a maximum of 18 per cent in Dog 7 on the fourth day.

Each of these dogs exhibited eosinophilia after intubation, and developed a perfect pulmonary emphysema. Shortly after intubation fine whistling râles could be heard all over the chest, which were accentuated by exercise and

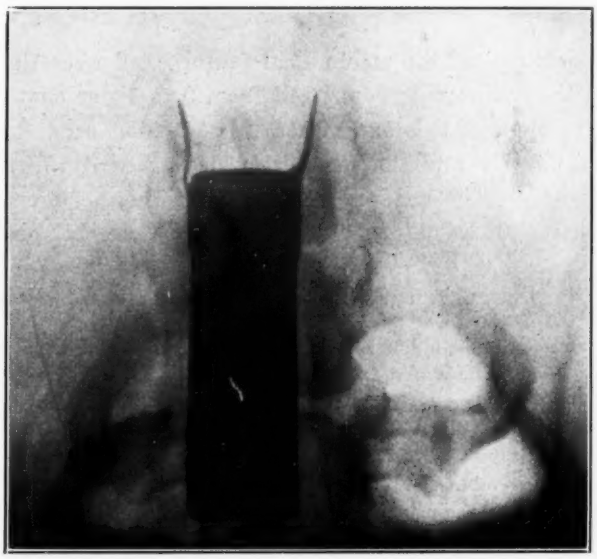

Fig. 1.—Dog 6, weight 13 kilos. Anteroposterior view of intratracheal ball valve in position.*

inhalation of irritants. This would indicate the added factor of bronchospasm, the total picture being identical with bronchial asthma. Of interest is the fall in eosinophile percentage in Experiment 6 following spontaneous extubation while physical signs of emphysema persisted. Reintubed this animal again showed an eosinophilia but of a higher grade. After exercise or inhalation of tobacco smoke, the eosinophilic count doubled; whereas, after extubation it remained at a normal level even though there were definite objective signs of emphysema.

The textbook[3] statement that eosinophilia accompanies emphysema did not hold true in Dog 6 following valve removal, even though all the desired physical signs of emphysema such as extension of the areas of pulmonary resonance and hyperresonant percussion note over both lungs were present. In this experiment the typical wheezing prolongation of the respiratory murmur dis-

*Ma. 10, Time 3 sec. Kv. P. 45 Screens double. Distance 20" Film Eastman duplitized.

TABLE I

TOTAL AND DIFFERENTIAL WHITE COUNTS ON INDICATED DAYS AFTER INTUBATION

DOG	DATE OF INTUBATION	CONTROL COUNTS					1					2				
		TOTAL	P*	L	O	E	TOTAL	P	L	O	E	TOTAL	P	L	O	E
1	Oct. 28	8500	71	26	3	0	12500	76	20	0	4	11800	74	20	0.5	5.5
2	Nov. 3	6200	68	30	1.5	0.5	14000	80	15	1	3	12600	78	17	0	5.
3	Dec. 2	7000	68	29	3	0	13800	78	18	0	4					
4	Dec. 8	3800	65	34	1	0	9000	70	25	1	4	13100	75	20	0	5
5	Jan. 10	7600	70	28	1	1	14800	75	21	1	3	15000	76	19	0	5
6	April 15	7200	72	27	1	0	12600	68	29	0	3	12400	65	30	0	5
	May 16 reintubed	7400	68.5	31	0.5	0	13700	71	24	1	4	14200	70	22	0.8	7.2
7	Nov. 19	9680	67.1	24.3	8.6	0	16480	65	28	0.6	6.4	13200	69.6	16.	1.	13.4
												14000 after	71.0 adren	15. alin no	1. change	13.0

*P, Neutrophiles
L, Lymphocytes
E, Eosinophiles
O, Other cells

No significant changes were noted in total number of red cells, or in the hemoglobin per cent.

appeared with removal of the mechanical factor; and with the return of normal expiration the eosinophile count fell from 9 to 1 per cent. The degree of emphysema developed in this experiment is shown in Figs. 2 and 3, the first taken before intubation and the second six days later following five days of expiratory difficulty. The autopsy report* of this experiment which follows is also negative for eosinophiles, but confirms the clinical picture of emphysema.

PATHOLOGIC FINDINGS

"Specimen consists of two lungs. They are pale, soft, and crepitant. The margins are ballooned out, almost white in color and the alveoli are quite visible as minute cysts. Scattered throughout the upper lobe is a rather irregular mottling of the same process. The middle lobe is normal except along the margins and the same is true of the lower lobe.

"The second lung composed of four lobes shows a similar process, the margins are pale, noncollapsed. The upper lobes show a spotty pallor which becomes less and less noticeable in the lower lobe where only the marginal portion shows this distended condition.

"Diagnosis.—Emphysema: Bronchi are distended, very pale, contain no visible mucus or blood.

"Smears from bronchi show little mucus, desquamated epithelial cells, few mononuclear cells containing pigment. Smears for bacteria were negative, Wright's smear failed to show eosinophiles.

"The striking lesion in the parenchyma is the irregular dilatation of the alveoli, which is best seen toward the peripheral portion of lung tissue. Here, the alveolar walls are extremely thin and anemic, and the alveolar spaces are three to four times as large as normal; bordering an area of alveolar dilatation are small patches of collapsed lung tissue.

"The bronchioles are distended.

"The walls of the vessels are normal except in a few branches of the pulmonary artery, which contain fibrin clumps that are undergoing organization.

"The glands along the bronchi and bronchioles as well as the mucosa are normal. The cilia stand out clearly, bathed by a very delicate layer of clear mucus.

"Occasional pigmented mononuclear cells are seen in the alveolar spaces and in the bronchial secretion.

"Differential count of white blood cells in large vessels shows normal count with no eosinophilia.

*For which we are indebted to the Department of Pathology.

TABLE I—CONT'D

TOTAL AND DIFFERENTIAL WHITE COUNTS ON INDICATED DAYS AFTER INTUBATION

3					4					5					6				
TOTAL	P	L	O	E	TOTAL	P	L	O	E	TOTAL	P	L	O	E	TOTAL	P	L	O	E
11800	74	21	0	5.															
13800	75	19	0.5	4.5															
11800	65	30	0.5	4.5	10900	65	31	0	4	11000	68	29	1	1	8000	70	29	1	0
	60	31	0	9						tubed	coug	hed u	p			70	27	2	1
13800	69	20	3	9															
14200	55	25.4	3.5	16.1	15400 after	56 adr	22 enal	4. n no ch	18.0 ange	12600	61	19	2.5	17.5	13000	60.5	21	1.5	17.0

P, Neutrophiles
L, Lymphocytes
E, Eosinophiles
O, Other cells

No significant changes were noted in total number of red cells, or in the hemoglobin per cent.

TABLE II

NO.	DIAGNOSIS	TOTAL W.C.	E	NECROPSY	AGE
1	Emphysema, chronic bronchitis, chronic myocarditis	11,680	4.8	Necropsy	71
2	Emphysema, carcinoma prostate	12,200	0.5	Necropsy	58
3	Chronic bronchitis emphysema	9,480	4.6		52
4	Bronchial asthma emphysema	8,800 to 20,400	4.5 to 20.0		34
5	Chronic bronchitis emphysema, lobar pneumonia	10,400 to 36,000	6.0 to 4.2		60
6	Emphysema, chronic bronchitis	11,200	3.8		57
7	Emphysema, chronic bronchiectasis	16,200	3.8		51

TABLE III

BLOOD COUNTS IN HUMAN SUBJECTS

NAME	BLOOD COUNT BEFORE DYSPNEA				EXPIRATION PARTIALLY OBSTRUCTED	BLOOD COUNT DURING DYSPNEA				BLOOD COUNT 5 MINUTES FOLLOWING DYSPNEA			
	N.	E.	L.	O.	MIN.	N.	E.	L.	O.	N.	E.	L.	O.
F. L.	62	0.5	32	5.5	1	71	2.0	24	3.0	66	0.5	29	4.5
M. H.	74	0	22	4	1	70	3.5	23	3.5	73	1	24	2.0
T. D. G.	66	0	30	4	2½	58.2	3.7	30	8.1	68	0	25	7
P. K.	68	0.5	25	6.5	1	69	4.0	23	4.0	70	1	26	3.0
K. G.	71	0	28.5	0.5	3½	66	4.5	26	8.5	70.5	1	28	0.5
M. W. B.	70	0	26	4	2½	72	6	20	2	71	0	25	4
E. S. R.	72.5	0	25	2.5	2	68	6.5	20.5	2.5	74	1	24.2	1.8
N. B.	71	0	26	3	3	66	6.5	24	2.5	74	0	23.5	2.5
P. B. H.	64	0	30	6	3	58.8	6.9	29.4	5.9	65	0	31	4
S. H.	66	1.5	30	2.5	1	71	8.0	19	2	70	2.0	26	2.0
L. S. N.	72	1	26	1	2	63	9.8	24.2	3	66.5	1.5	30	2
H. S.	74	1	24	1	3½	66.5	10.5	15.8	7.2	72	2.5	20	3.5
M. S.	72	1	25.5	2.5	2½	62	11	25	2	71	1.5	26	1.5
D. G.	73.6	0	22.7	3.7	2½	68	11	20	1	72	1.5	24	2.5
J. L. R.	70	1.0	21	8	3	58	14	22	6	67.5	3.5	20	9

"Examination of stroma surrounding bronchi and bronchioles shows no increase in cellular exudate and no infiltration of eosinophiles.

"Small fresh petechial hemorrhages are scattered throughout the lung parenchyma.

"Microscopic Diagnosis.—Parenchymatous emphysema, focal atelectasis; organizing thrombi in branches of pulmonary artery; petechial hemorrhages into alveolar spaces."

It is difficult to parallel this type of uncomplicated emphysema in human beings because such a condition does not call for hospitalization. Table II shows blood counts in selected cases. (All blood counts reported are averages of five or more taken at various times during the patient's illness.)

Fig. 2.—Dog 6. Anteroposterior view of chest before intubation, showing normal lung expansion.

In these cases of human emphysema all are complicated by pulmonary or bronchial pathology, with the exception of Case 2. This case was atrophic and of senile type, having no history of cough, and of interest since eosinophiles were practically absent in the blood smears.

The remaining cases all show a substantial eosinophilia. The high eosinophile count in the bronchial asthma case is explainable on the basis of bronchial spasm and expiratory dyspnea. In the remaining cases the emphysemas were complicated by chronic bronchitis coupled with a history of severe cough of long duration. During each cough there is a transitory overdistention of the lung, and this momentary obstruction to expiration is sufficient to cause both emphysema and eosinophilia and is similar to the eosinophilia and emphysema which we have produced experimentally.

In order to time the appearance of eosinophilia we studied the values produced by expiratory resistance upon medical students. Oral expiration was prolonged by employing a partially blocked flutter valve, the resistance offered varying from 20 to 30 mm. mercury pressure. Inspiration was through the nose. The results are tabulated in Table III.

The average time was two and one-fourth minutes, and the average degree of eosinophilia prior to and during dyspnea was 0.43 per cent and 7.19 per cent, respectively. In one-third of these cases the average eosinophilia was over 11 per cent. Hence, the eosinophilia occurring during dyspnea was approximately sixteen times greater than that prior to dyspnea.

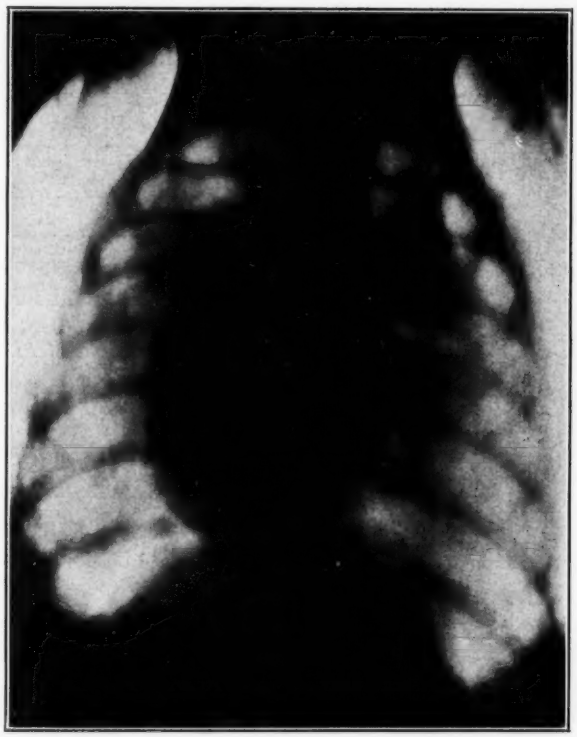

Fig. 3.—Dog 6. Anteroposterior view of chest six days after intubation showing extent of emphysema development.

We also believed it worth while to determine whether or not asthma, when temporarily relieved by adrenalin medication, was accompanied by changes in the eosinophilia count.

Table IV gives the results obtained in human cases, showing no eosinophile change under adrenalin. For completeness we have included negative results detailed in Table I, both as to spasm relief and eosionphilia change in dogs. One would expect lack of relief where mechanical conditions which from their nature and location would be the sole, specific and exciting cause of expiratory delay and alveolar distention.

TABLE IV
ADRENALIN ACTION IN HUMAN EOSINOPHILIA

PATIENT	HISTORY OF DISEASE	INITIAL COUNTS					ADRENALIN C.C.	COUNTS IN 15 MIN.					COUNTS IN 1 HOUR					COUNTS IN 24 HR.					COUNTS DURING REMISSIONS				
		TOTAL	P.	L.	O.	E.		TOTAL	P.	L.	O.	E.	TOTAL	P.	L.	O.	E.	TOTAL	P.	L.	O.	E.	TOTAL	P.	L.	O.	E.
1	Bronchial asthma. I. C. aged 34. Duration 8 yr. Attacks severe. Sensitive to wheat.	11,800	59.5	20	2.5	18	0.4 (no relief)	18,000	60	21	2	17	19,000	61	18.5	2	18.5										
							1.0 (relieved)	18,280	62	20	1.5	16.5	14,200	63	20	0	15.0	11,200	58	36	0	6	9,660	64	31	2	3
2	Bronchial asthma. V. M. aged 46. Onset after influenza 12 yr. ago. Attacks moderate.	12,600	68	22	1.0	9	0.8 (relieved)	13,000	66.5	23	1.5	9	11,000	68	22	1.5	8.5	8,800	65	30	0	5	7,860	66	28	4	2
3	Chr. eczema. J. A. C., aged 18. Duration 6 yr. Skin tests reveal hypersensitivity to egg yolks	8,200	65	30	0	5	0.8	8,200	64.5	30.5	0	5.5	8,060	65	29	1	5	8,400	66	30	0	4					

Camp[4] has reported that the eosinophiles are markedly increased by adrenalin dosage. Our investigation of this phase is negative both in human beings and dogs, and we are unable to account for his positive findings.

That the other human cases show an eosinophilia is explainable on the basis of accompanying respiratory difficulty.

The possibility of adrenalin raising the eosinophilic index had proved negative in our tests as shown in Table I, and also in Table IV.

In this short series of human cases of allergic disease accompanied by eosinophilia there was no fall in eosinophilic count many hours after adrenalin, even though the dosage was adequate to give symptomatic relief. This continued eosinophilia rules out the possibility of delayed adrenalin action.

DISCUSSION

Prior to the experiments of Pescatori,[2] Freedman and Jackson[1] showed that the CO_2 content of the blood and alveolar air is raised when expiration is partly obstructed, but these authors did not investigate the blood picture. One would expect such an increase in CO_2 tension when the retarded expiration was reinforced by an anesthesia.

The theory advanced by Pescatori, that the increase in eosinophiles is brought about by the above conditions, is based upon poisoning rabbits rapidly with CO_2 (asphyxia). By this method he obtained a high degree of eosinophilia, i.e., 35 per cent.

In the production of asphyxia there is a condition which closely parallels our mechanical retardation of expiration, because certainly the second stage of asphyxia is characterized by expiratory convulsions.

The cause of this sudden and apparently selective action accompanying bronchospasm, we believe, is due to the overdistention of the alveoli which, in turn, initiate reflex peripheral stimuli which brings about a release of eosinophiles from blood-forming organs. Garrey and Butler[5] believe a similar mechanism is accountable for the temporary leucocytosis resulting from sudden distention of the stomach.

The degree of individual response varied, as shown in Table III, but the striking phenomenon is that in all cases sudden definite eosinophilia developed. The rapidity of this eosinophilic release is indicative of reflex action upon blood-forming organs and blood reservoirs. The source of these eosinophiles is probably the reservoirs because of the rapidity of response and the maturity of the cells (multi lobulated nuclei).

The leucocytic increase in our experiments is definite. If there were nothing specific in the release of eosinophiles their increase should be proportional to the leucocytosis. But the percentage increase in leucocytosis is relatively small compared to the percentage increase of eosinophiles, which again suggests that a release of eosinophiles is probably due to some specific entity.

Additional evidence that CO_2 (chemical stimulation) is not the factor is to be found in the uncomplicated case of emphysema in Table IV: also in Animal 6, Table I, following extubation. In both of these cases the eosinophilic index is not raised although it has been shown by Dautrabande[6] and others that in emphysema there is an increase in CO_2 tension.

Recently Martin,[7] in his study of the effects of exercise upon leucocytosis, investigated also the reactions of the white cells under adrenalin. His results show that both exercise and adrenalin increase the total white blood cell count, principally the lymphocytes and the polymorphonuclear cells. Investigation of the effects of exercise upon the eosinophilic index was negative, although our total counts confirmed Martin's conclusions. Analysis of Martin's tables show that under adrenalin medication the greatest individual increase of eosinophiles was 1 per cent. In this case the initial count was 2 per cent before adrenalin and 3 per cent after. Other cases in his series show decreases under adrenalin, while the remainder show increases varying from 0.05 to 0.75 of 1 per cent. Certainly the minor increases reported by Martin could not be considered clinical increases, nor do we feel that his reported decreases up to 1½ per cent under adrenalin are of significance. One would expect just such slight variations in individuals.

As a result of our experiments a physiologic explanation is to presume that there is a temporary eosinophilia during the prolonged and forceful expiratory act, as for example, in whooping cough, blowing of wind instruments and glass blowing. That the data relative to eosinophilia in these conditions are lacking is explained by the fact that counts have not been carried out during the period of expiratory exertion.

It is an established custom for clinicians to associate eosinophilia as a definite blood finding of allergic phenomenon. We contend that the "allergic doctrine" confuses cause and effect, because in so-called allergic conditions there is frequently bronchospasm coupled with alveolar overdistention which produces eosinophilia. Eosinophilia, per se, does not suggest certain protein avoidance nor does it demand sensitizing tests.

CONCLUSIONS

1. Eosinophilia is produced by expiratory delay when accompanied by overdistention of the alveoli.
2. Evidence is offered which suggests that this response is specific and of a reflex nature.
3. Adrenalin does not affect the eosinophilic index.
4. The evidence available does not warrant the consideration of acidosis as a causative factor in eosinophilia.
5. Physiologic leucocytosis is not accompanied by eosinophilic increase.
6. Eosinophilia, per se, is not pathognomonic of allergic diseases.

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FURTHER STUDIES ON THE CULTIVATION OF *ENDAMEBA HISTOLYTICA* AND A COMPLEMENT FIXATION TEST FOR AMEBIASIS*

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I. CULTIVATION OF *ENDAMEBA HISTOLYTICA*

THE general belief that *Endameba histolytica* does not ordinarily ingest intestinal bacteria was contradicted by Boeck and Drbolav[1] on the basis of their cultural experiments. They showed that the amebas were able to ingest bacteria, and consequently, they believed that the organisms might thrive without access to tissue elements. The work of Dobell and Laidlaw[2] as well as Cleveland and Saunders[3] seemed to strengthen this contention. Dobell claimed that certain particular types of intestinal bacteria were especially favorable sources of food supply for the amebas. By the addition of such organisms, he succeeded in maintaining a cultural cycle of the amebas in vitro. Cleveland and Saunders cultivated the amebas from apparently bacteria-free amebic abscesses in the medium of Cleveland and Collier[4] and found that the amebas were able to multiply very rapidly when *Escherichia communior, Vibrio comma,* and *Neisseria catarrhalis* were present.

Though Kofoid and Wagener[5] believed that either coagulated egg or blood was required as a source of food for the amebas, Yorke and Adams[6] considered moisture and a suitable temperature (37° C.) to be the only essential factors in inducing excystation of the organism in vitro. They reported that excystation occurred when cysts were placed in either Locke-serum, broth, or physiologic saline and incubated at 37° C. for a few hours, but a total disintegration of the organisms occurred in these mediums after several hours' incubation. In view of these findings it occurred to the author that the addition of suitable substances to one of these media might result in the survival of amebas beyond the stage of excystation.

The purpose of this study is to describe a method of cultivation of amebas, worked out with these experiments as a background. The details of the method have been somewhat modified since the original report on the subject.[7]

The method developed consists of the inoculation of washed cysts into a medium tentatively called S. C. medium composed of nutrient broth and a mixture of starch and charcoal with and without Dorsett's egg medium.[8]

Constituents of the Medium.—1. *Nutrient Broth* consisted of peptone 10 gm., meat extract 3 gm., and NaCl 5 gm. in one liter of distilled water adjusted to P_H 7.0 and autoclaved at 15 pounds' pressure for thirty minutes. The P_H of the medium within the range of 6.8 and 7.4 also answers the purpose, though the optimum is found to be 7.0.

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I wish to express my gratitude to Doctor J. Bronfenbrenner for his valuable advice and criticism during the course of this study.

2. *The S. C. Mixture.*—This is a thoroughly triturated mixture of rice starch and animal charcoal in the proportion of 2:1 by volume. A small amount of the mixture is loosely placed in a small vial and sterilized by dry heat at 180° C. for forty-five minutes.

Rice starch provides the amebas with a definite source of assimilable carbohydrate and inhibits the growth of Blastocystis frequently met with in feces. The addition of rice starch by Dobell and Laidlaw to the original medium of Boeck and Drbolav has been generally considered as a decided improvement, as extremely small grains of rice starch are readily ingested by the amebas.

Animal charcoal is used to adsorb ammonia and hydrogen sulphide thus reducing their deleterious effects upon the amebas, and inducing a partial anaerobiasis in the medium by the adsorption of oxygen. The relative value of charcoals of various origins have been studied. Merck's purified animal charcoal and Norit were found to be superior to others for this work. The calcium phosphate contained in these charcoals acts as a buffer, and also helps to stimulate the metabolic activities of the amebas. It was found that the addition of excess calcium phosphate to the medium did not materially increase the growth of the amebas, but on the contrary stimulated a marked proliferation of the accompanying bacteria, so that the amebas were crowded out.

Preparation of Washed Cysts.—This is carried out best according to the method of Hegner.[9] Formed stool is thoroughly mixed by means of a sterile glass rod or spatula in order to obtain a uniform distribution of cysts. Liquid stool should first be sedimented, and the supernatant fluid carefully decanted. A portion of stool or sediment thus obtained is emulsified with a large quantity of water. This is filtered through four layers of gauze into a tall cylinder and allowed to stand for a few hours during which time cysts settle to the bottom. The process is repeated at least three times, until the sediment is found to be composed of the cysts and a small amount of débris. The sediment thus prepared, as a rule, contains fewer bacteria than are present in original stool. There is an especially noticeable reduction of the starch-splitting types of organisms, as is indicated by the presence of numerous unchanged starch granules in the medium after incubation.

When sufficient time is not available for the preparation of washed cysts, a portion of the sediment obtained from the preliminary filtration is transferred to a 15 c.c. centrifuge tube. By an addition of sufficient distilled water, this is washed by means of centrifugalization at 1,500 r. per minute for three minutes. This process should be repeated at least three or four times. Cysts, thus collected, were found to grow in the culture medium almost as well as those obtained by the slow sedimentation.

The author[10] recently found that washed cysts remained viable as long as nine days when left at room temperature (22° C.) and thirty-five days in a refrigerator. Furthermore, irrespective of the stages of development of these cysts, they were found to grow to maturity in vitro under favorable environmental conditions. Hegner, Johnson and Stabler[11] observed that immature cysts were able to excyst in the small intestines of monkeys within three hours after being introduced into the stomach. A successful cultivation in vitro,

therefore, appears to depend on the number and viability of cysts inoculated, but not upon the stage of development of these cysts as previously considered.

Primary Cultures.—At the outset of this investigation, it was thought that an introduction of a small bit of an infected stool into the medium was sufficient to induce a good growth of the amebas. Subsequent studies, however, indicated that washed cysts were superior for the cultivation irrespective of the number of cysts found in the original stool. Apparently, the growth of the amebas was prevented by multitudes of bacteria present in the original stool.

A small amount of washed cysts (0.1 c.c. or less dependent upon the number of cysts present) is introduced into 8 c.c. of the broth previously warmed to body temperature, and two 4 mm. loopsful of the S. C. mixture are added. The tube is now incubated at 37° C. After an incubation period of from twenty-four to forty-eight hours, the sediment is at first gently scraped off from the bottom of the tube by means of a pipette with a wide terminal opening, and then about 0.1 c.c. or less is withdrawn carrying with it minute particles of charcoal. The amebas are usually found adhering to the particles of charcoal at the bottom of the tube. The organisms, if few are present, therefore, may readily escape detection in a material without charcoal. The contents of a pipette are spread on a warm clean slide, covered with a cover glass, and examined by means of a 10 x eye piece and 16 mm. dry objective, preferably either on a warm stage or in a warm chamber. Some of the amebas, at first seemingly quiescent, soon begin to project their pseudopodia explosively. Within a few minutes some of them are found crossing the microscopic field with rapidity and in a definite direction in an apparent search for food. As a rule, within the cytoplasm of the motile amebas there are observed fewer starch granules than within the quiescent ones. The amebas, if kept warm and moist, exhibit markedly progressive locomotion, as observation continues. The addition of a small drop of 0.1 per cent neutral red helps to differentiate the ectoplasm from the endoplasm without affecting the motility of the amebas, and facilitates the examination greatly, as it gives the endoplasm a pinkish refractive tinge as contrasted with other objects in the microscopic field. Care should be taken to guard against air currents and desiccation of the specimen, as this may interfere with the motility of the amebas, thus inducing them to round up and die.

By the use of this medium, the amebas may often be seen at various stages of development; excystation, metacystic development and encystation as well as division frequently occur simultaneously. The extent of growth of the amebas in this medium is very striking. Thus, by the introduction of 80 cysts, the number of the trophozoites was calculated to be 21,200 after forty-eight hours' incubation in one instance, while, in other instances, 40 cysts gave rise to 13,900 and 72 cysts to 12,200 trophozoites.

While the medium seems to be very favorable one for *E. histolytica,* it does not seem to support the growth of other protozoa. Repeated attempts to grow *E. coli, E. nana,* and *I. williamsi* in this medium were without results. The same also holds true to a certain extent with the flagellates such as *Trichomonas hominis* and *Chilomastix mesnili;* these organisms grow quite readily in the absence of the S. C. mixture from this medium. Though no plausible explanation can be given at this time, it is suggested that P_H of the medium and the

nature of the bacterial contents, as well as the oxygen tension may not be suitable for these organisms.

Subcultures.—Subcultures may be made by inoculating a small amount of the sediment from the primary culture to a fresh tube of the original culture medium previously warmed to body temperature. Transfers should be made at least every forty-eight hours in order to induce a good growth.

It has also been found that when about 5 c.c. of the S. C. medium is superimposed on a slant of Dorsett's medium, an efficient medium for subculturing and primary cultures* is obtained. With careful manipulation, this medium serves to maintain luxurious growth of the amebas through many generations when transfers are made at twenty-four-hour intervals. The amount of the inoculum should not be greater than 0.05 c.c., as the excess often leads to the overgrowth of the accompanying bacteria which interferes with the optimum cultural environment for the amebas.

Maintenance of Cultures.—When washed cysts are first exposed to the action of a mixture of equal volume of gentian violet and acriflavin (1:100 dilution each) at 37° C. for one hour, and then inoculated into the S. C. medium on Dorsett's medium slants, the amebas usually maintain growth for as long as eight days without further transfer. In these circumstances the lag period before detectable evidence of growth is prolonged, but the development thereafter is remarkably luxuriant. The long lag period may be accounted for by the amebastatic activities of these dyes, such as had been observed by Churchman[12] in the study of bacteriostasis. The detailed study of this problem will be reported at a later date.

Diagnostic Value of the Culture Method.—The superiority of the cultural method over the direct microscopic examination and concentration methods for detection of amebas in stools was clearly shown by the fact that, of 346 stools examined at random for intestinal parasites, 5, or 1.44 per cent were positive for *E. histolytica* by the direct microscopic examination of the stools, 7, or 2.02 per cent, by the concentration method of Rivas,[13] and 9, or 2.60 per cent, by the use of this medium. In each instance, the final verification of the organism was made by permanent preparations which were stained by the method recently described by the author.[14] The cultural method, if properly employed, therefore, is of considerable value in the routine diagnosis for the presence of *E. histolytica.* Furthermore, the fact that other endamebas failed to grow in this medium adds to its value for diagnostic use.

II. COMPLEMENT FIXATION TEST FOR DIAGNOSIS OF AMEBIASIS

Craig[15] has reported positive complement fixation tests in 92 per cent of a group of 84 cases of amebic infection, all of which showed the amebas in the stools. Recently, Keifer,[16] aided by Craig, reported strongly positive complement fixation reactions with the same antigen (prepared from amebic culture in Boeck and Drbolav medium) in 15 out of 19 cases of chronic ulcerative colitis, but neither feces nor materials obtained by means of the proctoscope revealed the presence of the amebas in these cases. The question naturally arises as to the

*See additional note in abstract, Further Studies on the Cultivation of Endameba histolytica, J. Parasit. 20: 143, 1933.

significance of the positive reactions in these instances. They might conceivably be due to the presence of amebic lesions outside of the intestine. It is possible, though unlikely, that the positive reactions were caused by existence of complement fixing antibodies in the patient's serum for the *bacteria* in the antigen preparation used. Keifer suggested the possibility that the pyogenic infection of the colon in these cases might be superimposed upon a preexisting amebic ulceration and cited in support of this the disappearance of symptom in some of his cases following antiamebic therapy. There is, however, no direct evidence that infection with the amebas was the causative factor in any instance. The beneficial effect of emetine therapy by no means constituted proof of amebic infection.

The purpose of the present study is to further investigate the value of complement fixation tests in diagnosis using as an antigen the culture in the medium devised by the author.

Reagents.—One of the obstacles in perfecting the complement fixation procedure is the difficulty in separating the amebas completely from the accompanying bacteria in vitro so as to prepare a specific antigen. The selection of material suitable for preparation of the antigen requires utmost consideration, particularly with reference to (1) the number of bacteria in the original stool in proportion to that of the amebas, and (2) the choice of a culture method which will further reduce the number of bacteria and will stimulate the growth of the amebas.

Antigen: For the preparation of the antigen, Craig[17] utilized the Boeck-Drbolav medium[1] containing 2 or 3 amebas per microscopic field, while Menendez[18] used the material containing 8 or 10 per high power microscopic field. Hage[19] in his attempt to confirm Izar's[20] work with the complement fixation reaction attributed his failure to a lack of sufficient number of amebas to be extracted for the aqueous antigen. Because of the small number of amebas present Craig used in his work an undiluted antigen. The number of the amebas in the antigen in my study was considerably greater than in those of Craig and Menendez; the average number per high power microscopic field being 54. Repeated titrations of the antigen indicated that dilutions lower than 1:100 showed anticomplementary property. One-fourth of the anticomplementary unit of this 1:100 dilution was employed in the test.

Owing to the presence of the amebas only on or below the level of charcoal in the S. C. medium, no preliminary centrifugalization was deemed necessary to obtain the material to be extracted. The supernatant fluid above the charcoal was carefully pipetted off. The sediments of at least 24 cultures including the charcoal and starch were collected and placed in a conical centrifuge tube with an addition of warm normal saline solution. This was repeatedly centrifuged at slow speed, until the sediment contained a comparatively small number of the accompanying bacteria. Usually, seven or eight centrifugalizations were necessary to accomplish this end. By following the method of Craig, to one volume of the sediment was added ten volumes of absolute alcohol. The mixture in a tube, hermetically sealed, was left in an incubator at 37° C. for fifteen days, having been shaken vigorously at least three times a day during this

period. At the end of the period, the mixture was filtered through a fine filter paper, and the filtrate kept in a refrigerator until used.

In addition to the above, a second antigen was prepared by evaporating 10 c.c. of the above extract in an incubator at 37° C. until 1 c.c. mark was reached, and the evaporated portions replaced with normal saline solution.

As controls, extracts were also prepared in a similar way from the accompanying bacteria in the culture medium.

These antigenic preparations were titrated about every three weeks in order to check their potency.

Other Reagents: The hemolytic system used consisted of a 10 per cent sheep cell suspension, guinea pig complement (40 per cent) and rabbit antisheep amboceptor.

Test Proper: In this study, the ice box method of complement fixation was employed. To 0.1 c.c. of unknown serum, previously inactivated at 56° C. for half an hour was added one-fourth of anticomplementary unit of the antigen, and 2 units of complement. The tubes were left in a refrigerator for eighteen hours. One-tenth cubic centimeter of 10 per cent red cell suspension and 2 units of amboceptor were then added to each tube, and the incubation carried out in the water-bath at 37° C. for half an hour. Controls were run with known positive sera from cases of proved amebic infection and with normal sera, and the usual reagent controls. In the earlier part of the work the patient's sera were tested in various dilutions. The final readings were made after allowing red cells to settle sufficiently to the bottom of the tubes. For this purpose, the tubes are usually left in a refrigerator for two hours after the removal from the warm water-bath.

RESULTS AND DISCUSSION

TABLE I

CORRELATION BETWEEN RESULTS OF COMPLEMENT FIXATION TESTS AND ORDINARY DIAGNOSTIC METHODS*

CLINICAL DATA	TOTAL CASES	COMPLEMENT FIXATION POSITIVE. ORDINARY DIAGNOSTIC METHOD POSITIVE	COMPLEMENT FIXATION NEGATIVE. ORDINARY DIAGNOSTIC METHOD NEGATIVE	COMPLEMENT FIXATION POSITIVE. ORDINARY DIAGNOSTIC METHOD NEGATIVE	COMPLEMENT FIXATION NEGATIVE. ORDINARY DIAGNOSTIC METHOD POSITIVE
No evidence of amebiasis	130	–	130	–	–
Amebic dysentery	6	5	–	–	1
Carrier of *E. histolytica*	8	8	–	–	–
Suspected amebiasis	4	–	–	4	–
Ulcerative colitis	5	–	5	–	–
Totals	153	13	135	4	1

*Includes microscopic concentration and cultural examinations of stool and of material obtained by proctoscope (in some cases).

The comparison of examinations of stools and materials obtained by proctoscope with the findings in complement fixation tests with *E. histolytica* are shown in Table I. Both the feces and sera were found to be negative in 135 instances. In 13 cases of diagnosed or suspected infection the results were

positive both in feces and serum, while 4 gave a positive serum with a negative stool and one a negative serum with positive stool finding.

Judging from these observations, a negative reaction would appear to be quite significant if the tests were employed in the routine diagnosis, and would be of value in eliminating the possibility of amebiasis in a given individual. However, if repeated examinations were carried out, the number of positive stool findings might have been higher than that reported in this study. The author[9] recently observed the presence of definite encystment cycle in a carrier of *E. histolytica*, as he had recognized in those of *Giardia lamblia*.[21] Owing to considerable fluctuations in the number of cysts discharged from day to day, it was suggested that the examination of stools be made on alternate days for a longer period, rather than six consecutive days as advocated by Dobell.[22] The necessity of frequent examination cannot be overemphasized, as is illustrated in the following instance:

In a patient giving the history of protracted diarrhea accompanied by the profuse discharge of blood and mucus, the repeated complement fixation tests were consistently positive. The repeated examinations of stools and of materials obtained by means of proctoscope failed to show any amebas until finally on the fourth examination (a warm specimen), motile amebas were detected. Among eight individuals free from clinical symptoms but known to be carriers of *E. histolytica* and giving positive complement fixation tests, the cysts were detected in the stools only after repeated examinations in some instances.

Of the 6 patients with amebic dysentery 5 exhibited strongly positive complement fixation reactions and the initial stool examinations revealed presence of *E. histolytica*. These patients showed ulcerative areas about the rectum and sigmoid which teemed with actively motile amebae ingesting many red blood cells. The remaining case of amebiasis (with positive stool) never showed any capacity to fix complement in repeated trials.

With respect to the 4 cases with negative stools but positive complement fixation reaction, the following possibilities may be considered: (1) the amebas might have become localized in the tissues only, while the intestinal lumen was devoid of their presence (such may occur in case of metastasis to other parts of body), (2) there may have been an insufficient number of stool examinations to reveal the amebas, or (3) the amebic lesions may have been too high in the intestine to permit detection by the proctoscope. Such lesions are frequent, judging from the works of Hutchinson[23] and Callender.[24] Clark[25] reported in his study of 186 postmortem examinations in the Panama Canal Zone that the ulcers were most frequently found in the cecum, the portion where the greatest stasis existed. In such, no ulcerations were detectable by the proctoscopic examinations. Low[26] claimed that the practical value of sigmoidoscopic diagnosis is very much limited, as autopsy often revealed dysenteric lesions in the cecum and other portions of the large intestine with no rectal involvement.

It is interesting to note that in the five cases diagnosed clinically as chronic ulcerative colitis both complement fixation tests and repeated microscopic examinations revealed no evidence of amebic infection.

In the majority of the cases showing positive complement fixation, there were concomitant intestinal protozoan infections with organisms such as *Giardia lamblia, Chilomastix mesnili, Endameba coli, Endolimax nana* and *Iodameba williamsi.* These, however, did not influence the serologic findings. Among those individuals giving negative tests, there were several who harbored intestinal protozoa other than *E. histolytica.*

The results of the complement fixation reactions carried out using bacterial extracts as antigen consistently gave negative reactions irrespective of the outcome of the reaction with the amebic antigen. This confirms the findings of Craig as well as Sherwood and Heathman.[27] However, the bacterial extracts prepared in this study cannot be considered quite adequate, since the bacterial flora is variable according to individuals, and from time to time in the same individuals. It is still possible that some of the positive fixations obtained in clinically negative cases are due to this factor alone.

Prior to carrying out the complement fixation tests for *E. histolytica,* the majority of the sera were submitted to Wassermann or Kahn test or both in the Clinical Laboratories of the Department of Internal Medicine. It was found that existence of syphilis did not affect the specificity of the test. This confirms the previous findings of Craig and also of Spector.[28]

COMMENT

Hegner[29] has recently suggested that there are at least several types among the amebas according to their sites of localizations, each differing in their morphologic and physiologic characteristics, and that the universal conception that the tissue amebas give rise to the lumen types appears to be erroneous. He based his opinion upon histologic studies of the infected intestine which revealed no apparent tissue invasion. Judging from these observations, the formation of antibodies in response to the parasitic invasion may be variable according to the type of the amebas in question. It may be that antibody response is lacking in the case of the lumen and stool types, and this may explain the single instance reported above in which there was a negative complement fixation reaction with a positive stool.

Craig[30] has emphasized the fact that carriers, although showing no apparent symptoms, nevertheless usually presented definite pathologic lesions in the intestines at autopsy. It is probable that this explains the positive results in the complement fixation reactions in the 8 cases reported in this paper.

SUMMARY

1. A new culture medium (S. C. medium) for *E. histolytica* is described. By virtue of its simplicity and apparent efficiency, it is suggested that cultures in this medium may be valuable as a part of the routine diagnosis of infections with *E. histolytica.* The culture medium when superimposed upon Dorsett's egg slant serves very well for primary and subcultures. The fact that in these mediums other endamebas fail to grow seems to be of decided advantage in diagnosis.

2. When complement fixation tests were carried out with an antigen prepared from amebas cultured in the above medium, the following results were

obtained: Of 153 individuals examined, 135 known to be free of amebic infection, all were found to give a negative serologic test. Of the remaining 18 cases, 8 were known carriers of *E. histolytica* and gave positive serologic reactions. Six were diagnosed as clinical amebic dysentery and exhibited positive serologic reaction except in one case in which repeated trials failed to show any capacity to fix complement. There were 4 cases of ulcerative colitis which showed positive serologic tests, but no amebas were detected in the examinations of stools.

Thus, a negative serologic test appears to be quite significant, and valuable for eliminating the possibility of amebiasis in a given individual. A positive serologic test also seems to be quite specific in demonstrating the presence of *Endameba histolytica.*

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A STUDY OF THE SALIVARY AMYLASE IN PATIENTS WITH PERNICIOUS ANEMIA*

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PATIENTS with pernicious anemia are known to be subject to glossitis and often have involvement of the buccal mucous membrane. Since the ducts of the salivary glands open into the oral cavity, it was felt that it would be of interest to determine the amylolytic activity of the saliva, in order to ascertain whether the salivary glands were involved similarly. Carles and Delmas-Marsalet,[1] in 1924, reported that the amylolytic power of the saliva was lowered in cachectic states, such as tuberculosis, and increased in patients with peptic ulcer, indicating a possible parallelism with the gastric juice acidity.

The gastric juice findings of the patients presented in this paper have been previously reported by Helmer, Fouts, and Zerfas.[2] All of the patients had an achylia gastrica after histamine stimulation and were clinically and hematologically typical of pernicious anemia. Healthy adults with normal gastric juice acidity after histamine stimulation were used for controls.

METHOD

The method employed consisted essentially in the digestion of a starch solution with saliva diluted to a suitable concentration and the determination of the glucose formed by the resulting hydrolysis. Results, satisfactory from the comparative standpoint, were obtained, using a substrate consisting of 5 c.c. of a 1 per cent starch solution, unbuffered, and containing no added activating substances. Samples of saliva were collected a minimum of two hours after the ingestion of food or sweets, the flow of salivary secretion having been stimulated by the chewing of paraffin. The saliva was then filtered and diluted to a concentration such that 1 c.c. of the solution incubated with the substrate for one-half hour at 40° C. would hydrolize between 0.4 mg. and 1.5 mg. of

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glucose. The glucose content of the resultant mixture was determined by the revised method of Folin.[3]

The limits of true proportionality (0.4 mg. and 1.5 mg.) were determined experimentally by the digestion of 1 per cent starch with 1 c.c. samples of the same saliva diluted in the following proportions with water: 0.4 per cent, 0.6 per cent, 0.8 per cent, 1.2 per cent, 1.6 per cent, and 2.0 per cent. The amounts of glucose that were formed on hydrolysis are shown in Table I.

TABLE I

THE AMOUNT OF GLUCOSE PRODUCED BY DIFFERENT DILUTIONS OF THE SAME SALIVA

SAMPLE	PER CENT SALIVA	MG. GLUCOSE
1	0.4	0.41
2	0.6	0.50
3	0.8	0.68
4	1.2	1.01
5	1.6	1.41
6	2.0	2.06

Apparently the small amount of reducing substances ordinarily present in soluble starch had little influence on the resultant values, at least so far as a comparative effect was concerned, within this range, and it was unnecessary to consider a blank determination inasmuch as the same starch was used throughout.

Since the salivary amylase is so active, cotton plugs were placed in the pipettes to prevent contamination. The water-bath was carefully regulated, and the incubation was carefully timed from the addition of the enzyme to its destruction by the Folin-Wu copper sulphate reagent. The starch solution deteriorates rapidly at room temperature, so it was either made up daily or preserved with toluene, which did not interfere in the determination.

TABLE II

THE AMYLOLYTIC ACTIVITY OF THE SALIVA OF PATIENTS WITH PERNICIOUS ANEMIA AND OF NORMAL CONTROLS

	CASE	DILUTION OF SALIVA	MG. GLUCOSE	UNITS
Pernicious anemia	1	1.0%	1.61	161
	2	0.5%	0.65	120
	3	0.1%	0.75	750
	4	0.5%	0.68	136
	5	0.5%	1.04	208
	6	0.2%	1.24	620
	7	0.4%	0.65	162
	8	1.0%	1.68	168
	9	1.0%	1.17	117
	10	0.1%	0.67	670
	11	0.3%	1.22	406
	12	0.5%	1.31	262
	13	0.5%	1.25	250
	14	0.5%	1.42	284
Normal	1	0.5%	0.80	160
	2	0.5%	0.73	146
	3	0.5%	0.92	184
	4	0.3%	1.57	525
	5	0.5%	0.58	116

For means of comparison, a unit of activity was adopted, namely, the quantity of enzyme necessary to hydrolyze 1 mg. of glucose under the conditions described.

RESULTS

The results of the analysis are shown in Table II. Although the values varied enormously among different individuals, determinations on the same subject over periods of weeks demonstrated but slight variations. These results would indicate that the amylolytic activity of the saliva was not decreased in patients with pernicious anemia. Since all of these patients had an achylia gastrica, the degree of acidity of the gastric juice bore no relation to the activity of the salivary amylase. Furthermore, since patients with pernicious anemia have no free hydrochloric acid in their gastric juice, a considerable amount of starch digestion could take place in the stomach.

SUMMARY

1. A technic is described suitable for the quantitative determination of salivary amylase.

2. No demonstrable deficiency could be found in salivary amylase in patients with pernicious anemia.

The authors wish to express their thanks to Doctor L. G. Zerfas for his kind interest in the work.

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SICKLE CELL ANEMIA*

A Case Report

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THE importance of careful routine laboratory procedures is often minimized. In the case to be reported, the diagnosis, uncertain at first, and the explanation of several obscure physical findings, were at once made clear by the study of the stained blood smears taken by routine. Fradkin and Schwartz[1] say: "It is only by the accidental discovery of the sickle cells in the routine examination that the diagnosis is made." The word "accidental" should be unnecessary.

A brief review of the usual symptoms and findings in sickle cell anemia includes the following: (a) sickling of the red blood corpuscles; certain and varying numbers of the red blood corpuscles assuming a crescentic shape; (b) anemia, the hemoglobin usually averaging about 50 per cent and the total red blood corpuscles about 2,500,000 per c.c.; (c) the liver usually enlarged; (f) spleen, at first enlarged, a little later becomes fibrotic and smoother than normal; (g) trophic ulcers constantly seen; (h) heart murmur; (i) abdominal pain. Alden says the last is found in eight out of eleven published cases, while Hein and McCalla[2] list abdominal pain as the chief complaint.

Little is known as to the etiology of this peculiar disease. Eastland and Higgins[4] summarized the ideas of various workers, placing the blame for the changes in the morphology of the blood on: (1) primary faulty erythropoiesis;[5] (2) surface tension phenomena;[6] (3) immediate action of toxic, metabolic, or infectious agents upon a racial characteristic;[7] (4) changes in plasma;[8] (5) anoxemia.[9] The mechanism and the etiology remain obscure.

Since 1923 over 100 reports of sickle cell anemia have appeared in the literature. In many reported cases the sickle cell anemia was a secondary manifestation and the cause of death was certainly not the anemia. In some of these cases treatment failed to change the anemia, while the symptoms improved. Examination of healthy negroes showed as many as 16 per cent with the sickling tendency but without anemia. A study of autopsy material from the negro race by Rich[10] showed 62 instances of sickling in 5,000 autopsies, in none of which was the disease suspected. Furthermore, the sickling tendency does not seem peculiar to negroes, since Cooley and Lee[11] describe it in a family of Greeks.

Lawrence[12] reports three cases, not all negroes, in which the morphologic changes in the red blood cells were found but in which the classical picture was not so obvious. Recently, Terry, Hollingsworth and Engenio[13] presented

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more than 50 cases showing elliptic erythrocytes without any relation to age, sex, color, or blood group, and without relation to sickle cell anemia. A complete bibliography is given by Fradkin and Schwartz. The blood findings and the physical findings so often seen offer an interesting field for further study.

The treatment of sickle cell anemia is unsatisfactory. In spite of the therapeutic measures used, the disease usually proves fatal; few of the patients in the reported cases reaching thirty years of age. Among the procedures, splenectomy has been done with varying results. The use of splenectomy is based on the hypothesis that the red blood cell destruction takes place chiefly in the spleen, and upon the results in hemolytic jaundice. However, this procedure gives no assurance of help, although it tends to relieve the excessive red blood cell destruction. Stewart,[14] Bell and his associates,[15] Cooley and Lee,[11] and Hahn,[16] have reported cases in which splenectomy was done without cure but in which the excessive red blood cell destruction was lessened. Transfusions have been tried and invariably give only temporary relief. Liver therapy does not give a satisfactory response. The reasonable treatment consists of such supportive measures as generous amounts of wholesome food, rest, fresh air, and avoidance of infection, with blood transfusion and iron and arsenic to combat the anemia. Splenectomy should perhaps be advised as a last resort.

The differential diagnosis is sometimes difficult. Alden comments that tertiary syphilis obscured the diagnosis for months in one of his cases and that acute appendicitis was strongly suggested in another. Smith[17] reported a case in which congenital hemolytic jaundice was the tentative diagnosis. The enlargement of the heart with murmur often leads to the diagnosis of endocarditis. Probably the symptoms and findings most commonly suggest congenital hemolytic jaundice. However, in this latter condition leg ulcers and joint pains are rare, and the spleen is always enlarged. The laboratory findings of sickle cells and a normal fragility of red blood cells confirm the diagnosis of sickle cell anemia. Syphilis is readily ruled out with serologic tests. The diagnosis rests upon consideration of the blood picture, and the typical physical findings.

CASE REPORT

J. H., a negro boy, aged ten years, was admitted to the services of one of us, with a complaint of chronic ulcer of the leg. The ulcer was situated over the left external malleolus and had been present for over a month. There was no history of trauma. In the past this boy had been considered "delicate." He could not play with other boys of his own age because of dyspnea and shortness of breath. He had no illnesses but mumps in early childhood. No family history relating to his present illness could be elicited.

Only the positive findings on physical examination will be recorded. His sclerae showed a moderate jaundice. The heart was markedly enlarged to the left, and there was a loud systolic murmur heard over the mitral area, accompanied by a thrill. The liver was enlarged four fingerbreadths below the costal margin, and the spleen was not palpable. There was a small ulcer over the left external malleolus measuring 7 cm. by 4 cm., with its long axis vertical. The ulcer showed a granulating, unhealthy base covered by exudate. The edges were slightly undermined.

THE LABORATORY FINDINGS

Urine.—In several dozen specimens albumin was constantly present. Casts appeared at intervals and pus was always present.

Blood Count.—The red blood cells ranged from 1,500,000 to 2,260,000, and the hemoglobin remained about 45 per cent. The greatest improvement was seen temporarily following transfusion. Morphology of the red blood cells showed many "sickle" cells (Fig. 1) in both wet and stained preparations. This picture remained constant throughout the disease. Poikilocytosis and anisocytosis were always noted. The white blood cells totaled always about 10,000, a terminal count showing 27,000. The polymorphonuclear cells varied as the total white blood cells. Fragility tests showed a normal range. Bleeding time was one and one-half minutes, coagulation time two minutes, icteric index 20, and the van den Bergh a positive (faint) delayed direct reaction. Wassermann and Kahn reactions were negative. Blood cultures showed no growth. Blood chemistry: nonprotein nitrogen 66 mg.; creatinine, 1.3 mg.; sugar, 90 mg.; and chlorides, 460 mg. Blood group of the patient, father, and mother were (Moss) Group IV. Cross match was without change with the father. The father had a negative Kahn test.

Throat cultures were negative on two occasions. Stool negative on four occasions.

X-ray showed enlargement of heart to the left. Lungs and mediastinum were negative.

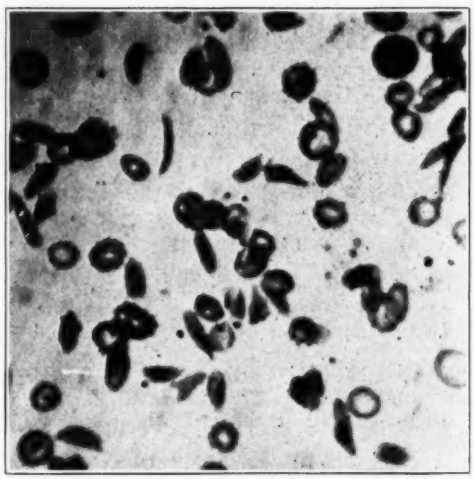

Fig. 1.—Showing the "sickle cells."

PATHOLOGIC REPORT

Gross.—Specimen consisted of a spleen, a small lymph node, and a piece of liver. The spleen was moderately enlarged and of the usual shape. It was firm in consistency and considerable fibrous tissue was noted throughout. Here and there were round, yellowish nodules of a soft consistency. The whole spleen was purple, mottled with a large amount of yellow. The lymph node measured only 1½ by 2 cm. and was soft and of a purple color. The small piece of liver showed nothing in the gross except that it was soft and red.

Microscopic.—Sections of spleen showed a thick, fibrous capsule. There was a large infarct, the center of which was filled with red-staining, necrotic material. Its margins were definitely fibrous and filled with chronic inflammatory cells. The pulp of the spleen was packed with red blood cells. In the interspaces were many "sickled" cells. Sections of liver showed areas of dilatation of the blood spaces. Here also were many "sickled" red blood cells. The liver cells showed everywhere clouding and granular changes in their cytoplasm. The interstitial tissue was moderately increased, and filled with chronic inflammatory cells. Sections of lymph node showed sinuses filled with blood, including many sickle cells. The follicles were small and widely separated.

Diagnosis.—White infarct of spleen, splenomegalia (sickle cell anemia), chronic hepatitis, chronic congestion of liver, hyperplasia of mesenteric lymph nodes, and chronic lymphadenitis.

TREATMENT

The treatment consisted of general measures, as rest in bed, sunlight, and nutritious food. The ulcer was treated by elevation of the part, and wet dressings, with no response. A transfusion, using the father as donor, was done. After several days some improvement in the boy's general condition, and in the anemia was noted. This improvement was only temporary and on the thirtieth day, after consultation, it was decided to do a splenectomy. The operation was uneventful, the spleen being removed without difficulty, and a wedge of liver taken. Unfortunately, postoperative pneumonia on the third day was followed by the death of the patient. Every effort to obtain an autopsy was combated by the parents.

SUMMARY

A brief review of the recent literature on sickle cell anemia is given and the outstanding symptoms and findings are outlined; the etiology being reviewed from the literature.

Sickle cell anemia being a rare and obscure disease, the treatment is largely symptomatic, and seldom successful.

The diagnosis may be made without difficulty. Too much emphasis cannot be placed upon the careful routine examination of the blood, which should include wet preparations as well as stained smears.

The case presented shows all the characteristic findings of sickle cell anemia. Transfusions and splenectomy failed to cure the patient.

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THE MECHANICS OF SPLEEN VISUALIZATION BY MEANS OF METALLIC COMPOUNDS, IN PARTICULAR THORIUM*

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EVAN SHUTE, M.D., CHICAGO, ILL.

SINCE thorium compounds such as "Thorotrast" are being increasingly used for spleen and liver visualization, we believe that all possible information as to their action and effects should be made available. Following the original reports of Radt[1] and Oka,[2] on the use of a colloidal thorium compound for spleen and liver visualization, we began some experimental work early in 1931 in an attempt to arrive at the exact method of visualization. The reports of these workers and R. Jaffe[3] gave the impression that the reticuloendothelial cells of organs, such as the spleen and liver, took up the thorium; which element was therefore directly responsible for the x-ray visualization that was obtained.

We used approximately the same thorium preparation as these authors had suggested. The method of preparation was essentially that described by Bluhbaum, Frik, and Kalkbrenner, quoted in detail by Radt. The actual preparation of our thorium compound was done by Dr. Marberg, a biochemist at the University of Chicago. It was prepared as follows:

> Freshly precipitated thorium hydroxide, prepared from 20 gm. of thorium nitrate, was washed free of electrolyte, suspended in 100 to 150 c.c. of water. The hydrosol was peptized by the addition of small amounts of normal hydrochloric acid added to the boiling suspension over a period of five hours. (Note: The use of the hydrochloric acid is a modification by Dr. Marberg, and we believe it makes a less toxic final production.) The final solution (150 c.c.) contains 20 gm. of thorium nitrate as Th $(OH)_4$. This is equivalent to 9.78 gm. of thorium or 12.5 gm. of thorium hydroxide. Also equivalent to 0.0653 gm. of thorium per c.c., or 0.0833 gm. of thorium hydroxide per c.c. The solution contains 0.0643 mols of hydrochloric acid per liter. Stable at boiling and refrigerator temperatures; also stable toward addition of neutral salts and glucose.

A dog two weeks old weighing 1.4 kilo was x-rayed and showed no spleen shadow. On Feb. 21, 1931, 1 c.c. of thorium solution, plus 7 c.c. of normal saline, was injected into the anterior fontanel with no clinical ill effect. A film taken twenty-four hours later showed faint visualization of the spleen. There was excellent visualization at forty-eight hours. At ninety-six hours the spleen shadow was beginning to fade. The liver was possibly denser than the other upper abdominal viscera, but could not be definitely differentiated. On Feb. 27, 1931, the same dose of thorium solution was repeated at the same site. Thirty hours later the spleen visualization was again at its maximum. The animal was killed and autopsied Feb. 28, 1931.

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Microscopic study of sections of the injected spleen showed irregular degeneration and cloudy swelling of its cells, especially adjacent to the sinuses and capillaries, the latter were crammed with thorium particles, and discrete brownish particles were scattered diffusely throughout the stroma. The corpuscles were relatively unaffected. There seemed to be no tendency for the ingestion of the particulate matter (thorium) by the reticuloendothelial cells.

We next used three dogs about two months of age but varying considerably in size. Dog 1 was a control, weighing 2,200 gm. The other dogs weighed 1,100 and 1,600 gm. respectively. All were x-rayed and showed no evidence of a splenic shadow. One cubic centimeter of a special colloidal solution of mercuric sulphite was administered intravenously to the 1,100 gm. dog. Films were taken twenty-four hours after injection and again at forty-eight hours; the spleen was not visualized. The 1,600 gm. dog was given 1 c.c. of the thorium solution plus 4 c.c. of a mixture of 10 per cent glucose and normal saline by the anterior fontanel. There was no systemic reaction. In twenty-four hours there was excellent visualization of the spleen, the shadow remaining the same density at forty-eight hours. All three dogs were killed within fifty hours from the beginning of the experiment. At autopsy the only gross change observed was in Dog 2 (the one injected with mercuric sulphite) whose spleen showed a purplish black discoloration.

Microscopically the lymphoid cells of the control spleen were normal. There were a few particles of hemosiderin here and there, but they were not dense.

The spleen of the "mercury" Dog 2 showed the cells crowded together with some swelling and pallor. There were much larger pigmented particles than the hemosiderin throughout the sections, but none of the large particles (mercuric sulphite) were inside the cells of the reticuloendothelial type. The blood vessels were filled with leucocytes and in many places contained much of this particulate material. In the spleen from the "thorium" dog there was swelling of the stroma and the cells were pale and swollen. The blood vessels showed the same type of leucocytic chemotaxis as in the "mercury" dog. The Malpighian corpuscles were not appreciably involved. No thorium had been taken up by reticuloendothelial cells but the stroma was literally peppered with thorium particles. A few macrophages were present and had ingested various quantities of thorium. Those that contained the greater quantity of thorium showed a marked degeneration.

It seemed from the above that simple cloudy swelling might prove to be an essential factor in visceral visualization of this type. Therefore, we undertook to study it in vivo. We gradually poisoned a three-month-old dog with a soluble salt of mercury (bichloride of mercury), giving 1½ gr. a day for three days, 7 gr. on the fourth day, and 7 gr. intramuscularly on the fifth. The spleen was visualized by x-ray on the fifth day quite clearly, and also on the sixth day, but perhaps a little less distinctly. The viscera as a whole seemed to show more definitely than usual. The dog was killed on the sixth day when acutely ill with mercurial nephritis.

Microscopically the spleen showed lymphocytes that were unusually large and crowded together. In fact the cells were so pale and swollen that it was difficult to differentiate lymphocytes from reticuloendothelial cells, monocytes, and etc.

RÉSUMÉ

The method of Oka and Radt for visualizing the spleen by the administration of thorium intravenously seems to be adequate as far as x-ray findings are concerned. However, the thorium seems to produce an intense degeneration of the spleen in doses too small to give clinical signs. The thorium appears to be taken up largely by the blood sinuses and lodged in the stroma rather than in the reticuloendothelial system. Other colloidal particulate matter (mercuric sulphite) is taken up in the same way but does not produce x-ray visualization of the spleen apparently because cloudy swelling does not result.

The spleen is visualized if an acute cloudy swelling is produced as in bichloride of mercury poisoning, but this visualization is not as marked as that obtained with thorium.

CONCLUSIONS

1. Intravenous injections of thorium give satisfactory x-ray visualization of the spleen.

2. This visualization of the spleen is due in part to the thorium particles in the stroma and capillaries, but cloudy swelling seems to be also an important factor.

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A STUDY OF PUNCTATE STIPPLING AS FOUND IN THE LEAD POISONING OF WILD DUCKS*

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PUNCTATE or basophilic stippling of erythrocytes has apparently been a subject of controversy since it was noted by Ehrlich in 1885.[1] Stippling was first correlated with lead poisoning by Behrend some fourteen years later, in 1899.[2] A voluminous literature on the subject accumulated, which when summarized by Pappenheim[3] in 1919, fell far short of proving the mechanism of its production, or of a clear-cut clinical conception of its significance. The toxic conditions in which stippling was found were well known; but regarding the granules themselves, opinions varied from the toxic granular degeneration theory of Pappenheim to the nuclear fragmentation theories of Kreibich[4] and Koch.[5] Many of our standard textbooks still quote one or the other of these theories, which, in fact, are both undoubtedly wrong.

Modern knowledge regarding this phenomenon began with Key in 1924.[6] Working with rabbits in experimental lead poisoning he proved that the percentage of stippled cells rose and fell in direct proportion to the reticulocyte variation, and that the number of reticulocytes was approximately equal to the combined number of stippled and basophilic erythrocytes, thus proving that stippled cells were immature erythrocytes. Four years later Brookfield[7] confirmed this finding in human subjects.

Cooke and Hill[8] in a study of the microscopic picture of pernicious anemia state that stippling is produced by the remains of the original cytoplasm of the erythroblast; and that the reason for its taking the azur-eosin stain after fixation with alcohol may be found in a defect in the immature lipin covering of the cells. Lane[9] shortly thereafter agreed with Cooke's hypothesis and further disproved the old theories by an ultramicroscopic (dark-field) study of stippled cells, the granules of which could not be demonstrated in wet or wet-fixed films. As all other toxic granular degeneration products or nuclear fragments can easily be demonstrated by strong indirect illumination, it seems thoroughly proved that the accumulation of basophilic staining material into clumps in certain young erythrocytes represents an alteration of the protoplasm of these cells that interferes with its ability to remain diffused throughout the cell during the process of drying.

Incidental to a survey of wild fowl in Louisiana to determine the occurrence and extent of accidental lead poisoning from ingested shot, a careful blood survey was added to the routine pathologic examination. Numerous specimens showing all degrees of stippling were encountered. The presence of nucleated erythrocytes in fowls enables a direct determination of the degree of poisoning

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necessary to produce stippling. The occurrence of basophilic stippling in large numbers of ducks is also of interest in view of the statement by Key[6] that several investigators, including A. S. Minot, had been unable to produce it in chickens.

Wild duck arriving in southern Louisiana in mid-November congregate on large shallow lakes formed by inundation of former agricultural reclamation projects in the fertile marsh areas. Members of the scaup group, particularly black duck and d'eau gris, in feeding on the seeds of an aquatic plant which are about the size of ordinary lead shot, find and ingest large numbers of lead pellets

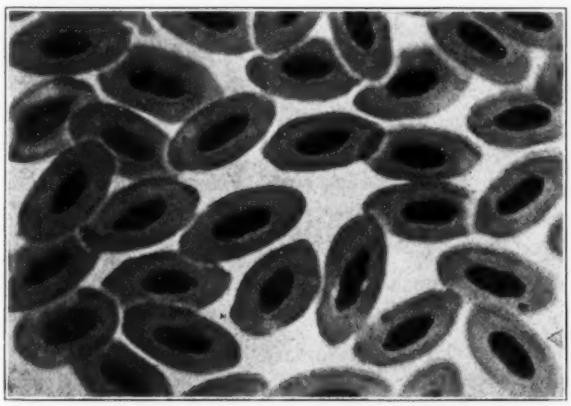

Fig. 1.—Normal blood of wild duck.

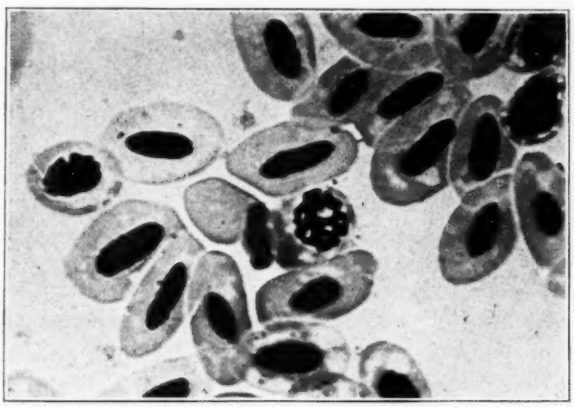

Fig. 2.—Active blood regeneration following acute blood loss in acute lead poisoning. Numerous shot present in the gizzard that had lost only a fraction of their lead content.

that have accumulated during the past few years of intensive hunting. Wetmore[10] presents a clinical and experimental study of this lead poisoning, citing experiments proving that three or four No. 6 shot will usually kill a captive wild duck. An average incidence of 12 shot were found in 89 ducks of this species in this investigation, the individual variation being from 0 to 140 shot.

Duck arriving in the ponds relatively free from lead (see Fig. 1) acquire their lead in apparently a few days' feeding. Within a week, and while still able to be taken on the wing, they present all of the symptoms of an acute lead

poisoning (Fig. 2) with tremendous loss of weight, anemia, diarrhea, bile-stained mucosa of the gizzard, active hemopoiesis of the long bones of the leg and an enormous increase in the number of reticulocytes, which may reach a total of 70 per cent of the cells in the peripheral circulation, together with large numbers of erythroblasts and megaloblasts. Those that survive this acute poisoning, as shown by the presence of numerous shot in the gizzard, and which have lost from 20 to 50 per cent of their former weight, now begin to show numerous stippled cells (Fig. 3) coincidental with unmistakable changes in the nucleus, indicating cell death. The nuclei of many of the slightly basophilic erythrocytes become swollen and pale when stained with Wright's stain. Stippling appears, the nucleus changes position to one side or one pole of the cell. In those cells with the largest basophilic granulation, the nucleus appears only as a shadow. Fragmentation of the nucleus can be observed as a terminal process in these stippled cells, the nuclear fragments staining a definite purple and the basophilic granules a deep blue with the polychrome stains. It would seem reasonably certain that the direct combination of lead with phosphates on the

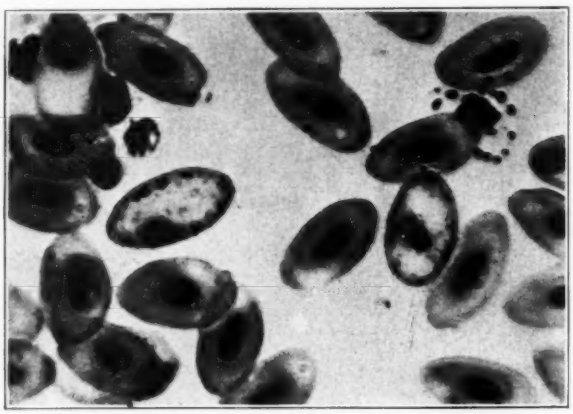

Fig. 3.—Chronic lead poisoning with stippling and nuclear degeneration and fragmentation. Numerous shot present in the gizzard that had been ground down to show a loss of 3 or more grams of metallic lead.

surface of the erythrocytes and the local liberation of a weak acid, as shown by Aub, Reznikoff and Smith,[11] is sufficiently toxic to produce actual cell death. The original cell protoplasm, represented by the basophilic stain of the erythrocyte, is not then able to withstand even the momentary effect of still further injury in drying, but precipitates or condenses into a granular form. It is thus demonstrated that the significance of a stippled cell is that of a *dying* cell and not merely a young cell that may continue to mature in the circulation. This visual proof of the chemical selection and untimely destruction by lead salts of a semimature cell is direct proof that stippling *is* a pathologic change in the sequence of events in the usual maturation of such cells in the peripheral circulation.

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LABORATORY METHODS

A NEW SYPHILIS REACTION, THE M.K.R. II IN CEREBROSPINAL FLUIDS*

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THE procedure for using the M.K.R. II in *serum examination* as now widely practiced may be briefly summarized as follows: 0.2 c.c. active serum is mixed with 0.5 c.c. of a freshly prepared suspension of the blue M.K.R. II antigen, and the test tubes are well shaken. For reading the results there is a choice of four different indicators:

1. *Macroscopic flocculation reaction:* The flocculation is read like an agglutination test after one and one-half hours with a hand lens.

2. *Microscopic flocculation reaction:* Immediately after setting up the tests drops of the reaction fluid are taken with a pipette and deposited on level slides which must be kept in a moist chamber for one hour, whereupon the reaction is read under a microscope with sixty fold magnification.

3. *Clarification reading:* The test tubes are left overnight at room temperature and the degree of clarification in each tube is then ascertained with the naked eye.

4. *Centrifugal technic:* Immediately after setting up the tests, the test tubes are centrifugalized for ten minutes. When they are taken out of the centrifuge, the supernatant fluid is carefully poured off and the tubes placed upside down in a rack. They are examined in half an hour's time to see whether the sediments have run down the sides of the tubes, or whether they have remained unaltered.

In the course of *experiments with cerebrospinal fluids* which I carried out jointly with Dr. Holthaus, it soon became apparent that the dosage of the serum test and the various methods of reading it could not be applied without some modification to the investigation of spinal fluids. Weak positive spinal fluids, in the dosage 0.2 c.c. fluid + 0.5 c.c. antigen suspension, were found to give a negative reaction. Thereupon we increased the amount of fluid, at the same time decreasing the amount of antigen, for instance: 0.5 c.c. spinal fluid + 0.1 c.c. antigen suspension. It now showed itself however that, owing to the small amount of antigen, the reaction fluid was not turbid as in the serum test, but more or less clear from the outset, so that there could be no question of applying the clarification indicator to the readings. But neither were the flocculation reading or the centrifugal technic quite satisfactory.

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We were therefore under the necessity of searching for a *new indicator* for *spinal fluid examination;* this we eventually found in *sediment reading.* If, after setting up the tests, the test tubes are left standing overnight at room temperature, a deposit of sediment becomes visible in each tube presenting variations in the case of negative or positive reactions which are easily recognizable with the naked eye. In *negative* reactions a blue, fairly clean-cut disc-shaped sediment forms at the bottom of the tube. In *positive* reactions there is no sign of this blue disc, but the bottom of the tube is covered with a light, whitish, filmy sediment, sometimes so slight as to be only distinctly ascertainable as sediment with the help of a hand lens. *The determinating factor for the appreciation of the reaction is always the presence or absence of the characteristic blue, disc-shaped sediment. If this is visible, the reaction must be marked negative; if not, the result is positive.*

TECHNIC OF THE SPINAL FLUID REACTION

1. *Reagents.*—The same M.K.R. II Standard Antigens are used as for the serum tests. Also the 3.5 per cent salt solution of the serum tests is used in this reaction; for this an absolutely pure preparation is necessary, viz.: natrium chloratum pro analysi.

2. *Pipetting the Spinal Fluids.*—Small *test tubes* about 8 cm. in length and as exactly as possible 1 cm. in diameter (so-called M.K.R. tubes) are used; they should be uniformly well rounded at the base. The tubes are placed in a perforated tube rack. Care must be taken that the perforations in the lower ledge of the rack be of a size which, while preventing the test tubes from falling through, leaves as much as possible of the surface of the bottom of the tubes open to examination from beneath through the holes in the rack.

The *spinal fluids* must be examined in an *active* state. It is absolutely essential that they are *thoroughly centrifugalized* so as to be entirely free of any corpuscular admixture. When the spinal fluids are sent to the laboratory from a distance, it is most important that the containers should *not be closed with cork, but with rubber stoppers.* Nonspecific reactions may sometimes be occasioned by the dissolving of certain substances during the transport when cork stoppers have been used.

Each spinal fluid is operated in 3 tubes: in the first, 0.5 c.c. fluid, in the second, 0.2 c.c., in the third, 0.1 c.c.

3. *Preparation of the Antigen Suspensions.*—The requisite quantity of standard antigen* for the tests is pipetted to the bottom of a large test tube

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PREPARATION OF THE M.K.R. II STANDARD ANTIGEN

**Heart Powder.*—The antigen is made from perfectly fresh beef hearts. The hearts are freed from fat and sinews, so that the pure heart muscle is left. This is put through an ordinary meat mincer, and then the finely chopped-up heart muscle is spread out in a flat layer on glass dishes; these are placed in a drying apparatus at from 40 to 50° C. The drying is continued until the heart pulp separates from the glass in hard crusts. These crusts are completely scraped off the glass dishes with a knife, and then placed in the incubator at 37° for two days, in order to secure its being dried as thoroughly as possible. The relatively large hard crusts of the dried heart muscle are reduced to small pieces in an absolutely dry meat mincer, and are then put through a coffee mill, so that an extremely fine powder results. This is poured into dry flasks which should be well stoppered and kept in a cool room. In making the antigens, it is advisable to mix the powder of several different hearts, because the individual hearts show slight differences in their lipoid content.

Ether Extraction.—Put 10 gm. of the heart powder into an Erlenmeyer flask of 100 c.c. content, or into a bottle of the same size. The glass vessel must be closed with a stopper covered with tin foil of good quality. Extract the powder first with ether and afterward with alcohol. Use exactly 70 c.c. ether to 10 gm. of powder; shake it up several times, and let the

or pointed glass vial. The tenfold quantity of 3.5 per cent salt solution is put into a second tube or vial of the same size. Antigen and salt solution are heated *separately* in a water-bath at from 57 to 58° until they have reached a *temperature of 55-56° C.* (about 10 minutes). The right temperature may be easily ascertained by means of a thermometer which has been placed at the same time in the water-bath in a vial filled with ordinary water. The level of the water in the water-bath must be 1 or 2 cm. higher than that of the salt solution in the vial in order to ensure uniform heating. If there is not a regulated water-bath at disposal, an ordinary tub may be filled with water at from 60 to 62° C. and, when the vials and control glass have been put in, left to cool down naturally to about 57° C.

After this preliminary heating of the reaction fluids, the salt solution must be poured rapidly into the vial containing the antigen, the mixture then poured back into the empty glass and back again into the antigen glass. *The mixing must be executed rapidly,* but care must be taken to avoid spilling any of the fluid during the manipulation. The freshly prepared antigen suspension is of a milky turbid bluish hue; it must be put back *at once* into the water-bath for *exactly two minutes* for *maturing,* and then utilized immediately for the tests.

In most laboratories the examination of spinal fluids will be only a fraction of the serum examinations. The antigen suspensions for sera and spinal fluids being identical, *the same antigen suspensions are of course used for both,* and it is not necessary to prepare them especially for spinal fluids.

4. *Mixing Spinal Fluid and Antigen Suspension.*—To the first tube with 0.5 c.c. spinal fluid, 0.1 c.c. antigen suspension is added; to the second with 0.2 c.c. spinal fluid also 0.1 c.c. antigen suspension, and to the third with 0.1 c.c.

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vessel stand for two hours, shaking several times again during this period. Filter off the ether through an ordinary paper filter, and again add 70 c.c. of ether to the powder. Proceed then in the same way as before for two hours. Filter off the ether once more. For the third time, add 70 c.c. of ether to the heart powder. Shake up several times during the next two hours. Afterward let the vessel stand undisturbed until the following day, and then filter the ether off. On filtering the whole powder is as far as possible, put on to the filter. After the fluid has passed through to the last drop, the filter should be carefully spread out flat and placed in the incubator at 37° until the next day. The glass vessel in which there still remain traces of the powder, should likewise be placed in the incubator. By the following day, the ether will have evaporated for certain. Put the powder on the filter back again into the bottle, and begin with the alcohol extraction.

Alcohol Extraction.—Pour 80 c.c. of 96 per cent alcohol over the heart powder which has been collected as completely as possible in the glass vessel. Let the bottle stand for eight days at room temperature, thoroughly shaking it several times a day. Then filter off and keep the heart extract at room temperature.

Adding of Balsam of Tolu and Victoriablue.—Dilute 10 c.c. of the heart extract with 90 c.c. of 96 per cent alcohol and add 1.4 gm. of balsam of Tolu to this dilution. Place the bottle in the incubator at 37° for two days and shake it several times a day. Then keep it at room temperature for a week and filter off through an ordinary paper filter. After adding of 0.01 gm. of Victoriablue to the filtered Tolu-Antigen it is ready for use.

Standardization of the M.K.R. II Antigen.—The Antigen thus prepared possesses approximately the right lipoid content of 1:10, requisite for the syphilis tests. In order to be able to weaken or to strengthen it, as the case may be, some balsam of Tolu in the proportion of 1.4 per cent is dissolved in the undiluted alcoholic extract which is rich in lipoids, and some also in an extract diluted 1:100 with alcohol which is extremely poor in lipoids. The addition of the undiluted extract to the M.K.R. II antigen serves to increase its lipoid content and thus to weaken the sensitivity of the tests; the addition of the much diluted extract, to lower the lipoid content and thus to make the tests more sensitive.

The standardization of the antigen must be performed, in testing it with positive and negative sera, according to the prescriptions for the M.K.R. II (Zentralbl. f. Bakteriol. 125: Pt. 1, 1932). In case the results in the serum tests should be somewhat too strong, and there should be a tendency to unspecificity, the lipoid content of the antigen must be slightly increased. If, on the contrary, the results are too weak, as compared with other syphilis reactions or with the results obtained with a Standard M.K.R. II Antigen, the lipoid content of the antigen in question has to be somewhat reduced.

All antigens should be kept at room temperature (not in the refrigerator) in well-stoppered bottles protected from light. The extract bottles must be stoppered with corks which should be covered with tin foil of good quality. If antigens are forwarded from one laboratory to another in frosty weather, it is advisable, on their arrival, to place them first in the incubator at 37°, and only then to store them at room temperature.

spinal fluid, 0.2 c.c. antigen suspension is added. The racks are well shaken after the addition of the antigen suspension.

If it is desirable to appreciate the quantitative degree of weak and medium strong positive reactions *with great exactitude,* two further tubes may be added to the original three. To this end another tube containing 0.4 c.c. spinal fluid and 0.1 c.c. antigen suspension is inserted between the first and second, and between the second and third a tube with 0.2 c.c. spinal fluid and 0.2 c.c. antigen suspension.

5. *Reading the Results.—The tests having been left standing overnight (sixteen to twenty-four hours) at room temperature of about 20° C.,* protected from cold draughts, the results are read with the *naked eye.* The investigator stands in front of a well-lighted window or an artificial source of light above his head, lifts the racks carefully, and holds them before him somewhat above his eye level, at the same time slanting them slightly forward. In this position the apertures of the test tubes are in a slanting direction to the source of light above and the base is toward the investigator.

a. *Negative Reactions:* In all three tubes *blue disc-shaped sediments* are visible in the center of the bottom of the tube, their coloring contrasting sharply with their surroundings. These blue sediments are *soft and have a tendency to dissolve.* If the rack is held in a slanting position for some little time, they will, following the law of gravitation, dissolve themselves into a thin streak. Shape, color and dissolubility of the sediments are the characteristics of a negative reaction.

b. *Weak Positive Reactions: The first tube with 0.5 c.c. of spinal fluid does not show any blue disc-shaped sediment.* The bottom of the tube is of a whitish opalescent hue. On close examination a very light filmy sediment is visible uniformly spread over the bottom of the tube. When held slanting for any length of time, this sediment does not dissolve; it will sometimes change its position as a whole, or it may crumble to pieces. *Both the other tubes present the characteristic blue negative discs.*

When in the first tube neither a distinct dissoluble blue disc nor the *uniformly* opalescent whitish coloration at the bottom of the tube is visible, the reaction must be marked *doubtful* (±). Then there is generally a rudiment of disc formation to be seen in the first tube with a number of minutest fragments of sediment strewn around it. In such cases the central disc is usually not soluble but adheres more or less firmly to the bottom of the tube.

c. *Medium Strong Positive Reactions:* The *two first* tubes with 0.5 c.c. and 0.2 c.c. spinal fluid show *the positive sediment* described in paragraph b., while the *third tube* has a *negative disc.*

d. *Strong Positive Reactions: The negative blue disc is not visible in any of the tubes.* They all show the connected whitish sediment of a positive reaction completely covering the bottom of the tube. In correspondence to the relatively larger amount of antigen in the last tube, the sediment in it is somewhat denser than in the first two, and may sometimes be slightly bluish in coloring.

e. *Hyperpositive Reactions:* Occasionally, though rarely, in cases of paralysis and tabes, spinal fluids may be observed which react so strongly that they

present an inhibition in the first or in both the first tubes. In these tubes more or less distinct blue negative discs have formed, while the characteristic broadly spread positive sediment appears in the last tube only. Compared to the weak positive reaction there is therefore in such cases a *reversed scale with the strongest reaction in the last tube.*

f. *Bacterially contaminated spinal fluids,* for instance, such as derive from cases of meningitis or have become decomposed during transport, may occasionally show the *same scale as hyperpositive reactions.* They must therefore be differentiated by a special test. To 0.1 c.c. of the questionable spinal fluid, 0.1 c.c. of a proved negative active serum and 0.5 c.c. of the usual antigen suspension is added. The mixture is well shaken and the reading performed in the way usually practiced in serum tests, viz.: either as macroscopic or microscopic flocculation or on the following day as clarification. *In the serum dilution contaminated spinal fluids always give a plainly negative reaction, whereas hyperpositive spinal fluids give a strong positive reaction.*

THE M.K.R. II AS COMPARED TO OTHER SPINAL FLUID REACTIONS

Our comparative investigations comprising some 600 cases have shown that the M.K.R. II applied to spinal fluids is appreciably superior in *sensitivity* to the Wassermann reaction, and is at least equal to the Kahn standard method and the Müller conglobation reaction.

Also with regard to *specificity* the M.K.R. II in spinal fluids is in no wise inferior to the above-named methods. It is a very simple method, is easily read, and provides the possibility of obtaining a good quantitative gradation of positive reactions with only three test tubes.

The M.K.R. II thus belongs to the group of particularly sensitive specific syphilis reactions in spinal fluid diagnosis as well as in serodiagnosis.

A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF BILIARY ACIDS IN BODY FLUIDS: WITH A NOTE ON THEIR ALLEGED PRESENCE IN NORMAL BLOOD*

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SINCE the introduction of the Pettenkofer[1] reaction for the recognition of bile acids, many attempts have been made to apply this test for estimating bile acids in blood. As a result of these researches it has been assumed that a positive Pettenkofer reaction given by specially prepared extracts of normal blood is an indication of the presence of bile salts. On this assumption Aldrich and Bledsoe[2] found that normal blood contains bile salts to the extent of 3 to 6 mg. per 100 c.c., while Charlet's[3] average findings are given as 3.5 to 3.8 mg. in normal rat blood.

On the other hand, by using different means of isolation but still using the Pettenkofer reaction, Perlzweig and Barron[4] as well as Gregory and Pascoe[5] deny the presence of bile salts in normal blood.

Recently Walker[6] has called attention to the interesting and important fact that the substance responsible for the color production in the Aldrich and Bledsoe process is cholesteryl oleate. This is hydrolysed by barium hydroxide to barium oleate. The cholesteryl oleate and barium oleate both give a positive Pettenkofer reaction.

From a practical standpoint, however, it cannot be denied that nearly all the methods have the common objection in that the Pettenkofer reaction is employed. A critical study of the quantitative aspects of this reaction has revealed many undesirable features in that: (a) the color reaction is not specific for bile acids, Mylius[7] finding 15 other substances (including barium oleate) that give a Pettenkofer reaction; (b) the quantitative recovery of bile acids is very variable with errors from 1 to 20 per cent; (c) the red or reddish color varies considerably as to color shade making comparison in the colorimeter exceedingly difficult.

It is obvious from the above results that the Pettenkofer reaction is wholly unsatisfactory for quantitative purposes even with pure solutions of bile acids, and quite unreliable even for qualitative work on body fluids.

A large number of tests have been advanced for the detection and estimation of bile salts in the urine, most of which again employ the Pettenkofer reaction, either direct to the urine, or to the isolated salts. If the reaction is applied direct to urine containing proteins a positive Pettenkofer reaction is always obtained. It hardly needs to be emphasized here that chromogenic substances yield intense colors with sulphuric acid. In the isolation method where the bile salts are precipitated from urine by saturation with magnesium sulphate,

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The studies were carried out in the Bio-Chemical Laboratory, Charing Cross Hospital, Institute of Pathology, by kind permission of Dr. J. Patterson, biochemist to the hospital.

bilirubin is also precipitated along with many other urinary pigments. As bilirubin is almost always present in combination with bile salts, the color produced in the final stage of the method is entirely lost in the dark brown or blackish mass obtained by the charring action of sulphuric acid on bilirubin and other urinary pigments. This process yields but little better results than that of the direct method. It is of great importance in patients treated with drugs which are potential liver poisons to try to obtain early warning of toxic action; again in patients with subicteric tints and fevers of unknown origin a reliable and quantitative method might prove of clinical value. Comparatively little work has been done in this direction, owing, presumably, to the methods being subject to the numerous limitations already mentioned.

The work outlined here was undertaken in order to arrive at a quantitative method for estimating bile salts. Various substances other than bile salts giving a positive Pettenkofer reaction and likely to be present in blood were subjected to the new reaction and found to be completely negative. The list includes cholesterol, cephalin, glycine, lanoline, phospholipines, lecithin, taurin, and oleic acid (present as cholesteryl oleate).

The following is the principle of the method:

The urine is acidified with hydrochloric acid and saturated with solid ammonium sulphate. The bile acids are precipitated completely, along with other urinary pigments. Bilirubin when present is also precipitated. To prevent the precipitated bile salts from adhering to the sides of the boiling tube and stopper, a small amount of protein is first added to the urine before adding the hydrochloric acid and ammonium sulphate. The protein has the effect of "holding" together the biliary acids, and thus overcoming great practical difficulties in that obvious loss of material is prevented as well as rendering time-consuming efforts unnecessary. The precipitate is filtered and washed with a saturated solution of ammonium sulphate to remove any soluble adhering pigments. The filter paper is carefully dried and the bile salts plus the insoluble urinary pigments extracted with absolute alcohol. A saturated solution of barium hydroxide is added to the extract, and after further concentration the mixture is diluted to a convenient volume with alcohol, and filtered after thorough cooling. The addition of the barium hydroxide renders bilirubin and other interfering urinary pigments insoluble in absolute alcohol. The alcoholic extract is evaporated to dryness and a reddish purple color produced from the residue by the action of levulose and pure hydrochloric acid. The standard is an alcoholic solution of sodium tauroglycocholate and is similarly treated. The colors are then compared colorimetrically.

Reagents and Apparatus.—

1. Pure concentrated (A. R.) hydrochloric acid (sp. gr. 1.16) HCl = 36.465.
2. Pure syrupy (A. R.) phosphoric acid (sp. gr. 1.75) H_3PO_4 = 98.051.
3. One per cent pure levulose (Merck) in a saturated solution of benzoic acid. This keeps indefinitely.
4. Saturated aqueous solution of ammonium sulphate.
5. Solid ammonium sulphate.
6. Fresh blood serum or plasma. For the precipitation to succeed it is absolutely essential that the serum or plasma employed be perfectly fresh, and this is usually obtainable in any modern laboratory.

7. Absolute alcohol (97 per cent).

8. Filter papers: Whatman extraction thimbles, size about 22 by 80 mm., and ordinary No. 1 papers, 7, 9, and 11 cm. in diameter.

9. A saturated aqueous solution of barium hydroxide.

10. Five-tenths per cent sodium tauroglycocholate in absolute alcohol. Owing to the hygroscopic nature of the bile salts, it is important to weigh out the above amount on glazed paper, and as quickly as possible. Transfer the bile salts to a 100 c.c. flask, add about 60 c.c. of absolute alcohol and immerse the flask with constant agitation in a boiling water-bath until the bile salts have dissolved completely. Cool, and dilute to the mark with absolute alcohol. After mixing thoroughly, filter through a 9 cm. paper.

11. Preparation of asbestos mat. Use a Gooch crucible of about 15 c.c. capacity (see Fig. 1) or an asbestos mat supported on a small (15 mm. diam.) perforated porcelain plate, resting in a conical funnel that passes through a rubber stopper fitting the neck of a filtering flask. The mat is prepared as follows: A suspension of well-washed asbestos is poured into the Gooch crucible or onto the plate and allowed to settle down without suction. After a short time the filtering flask is connected to the suction pump and the water pressure turned on. It is important not to suck too hard. The amount of asbestos required is such as to form a mat about 3 mm. in depth.

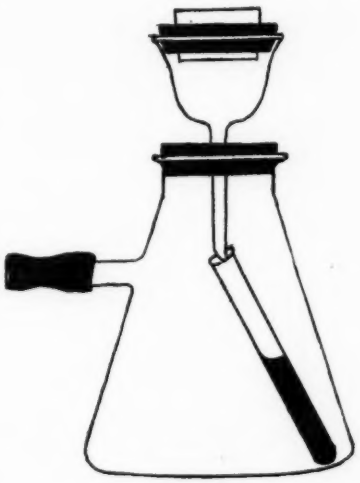

Fig. 1.

A small porous plate may be placed on top of the mat to prevent the latter from being disturbed too much by pouring on water or the phosphoric acid mixture. The mat is then washed two or three times with distilled water, gentle suction being applied after each addition. The final suction should be sufficient to make the mat quite firm. After being used for filtration of the phosphoric acid mixture the asbestos should be washed well with distilled water before further use.

METHOD

For Urine.—Introduce 25 c.c. of urine into a thick-walled boiling tube (Monax combustion tubes, 1 inch by 6 inches) followed by 0.05 c.c. of fresh blood serum or plasma. After mixing with the protein solution, add 2 c.c. of pure concentrated hydrochloric acid, mix again, and then saturate the acidulated solution by the addition of 25 gm. of solid ammonium sulphate. Securely stopper the tube, and after thorough shaking, stand for about ten minutes with occasional agitation to ensure for complete saturation with the ammonium

sulphate. Filter through a Whatman No. 1, 11 cm. paper which has been previously damped with a few drops of a saturated solution of ammonium sulphate. The filtrate should be transferred back to the precipitation tube and after mixing well, filtered again. This process prevents loss of any bile salts which may have escaped through the filter paper with the first few drops of the filtrate. The filtrate can now be discarded. The filter paper and precipitation tube are then washed with about 50 to 100 c.c. of saturated solution of ammonium sulphate, transferring about 20 c.c. of this at a time to the precipitation tube and after stoppering and shaking vigorously, poured on to the filter paper. In this manner traces of bile salts adhering to the boiling tube and the stopper are removed as well as the HCl introduced in the first stage of the process. Most

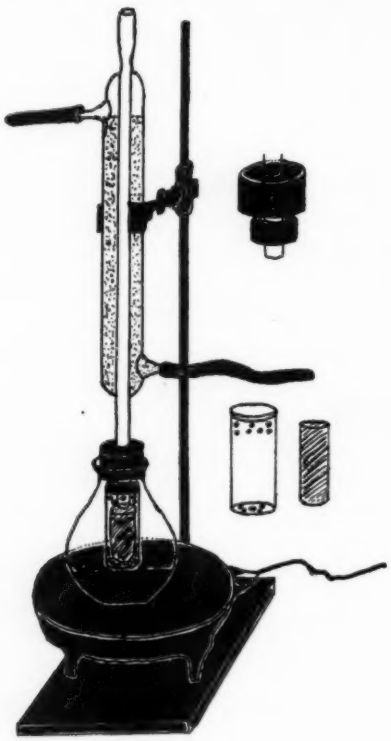

Fig. 2.

of the interfering urinary pigments are also removed by this washing. The washed paper and funnel are allowed to dry overnight in the incubator at 37° C. It has been found possible to dry the filter paper containing the bile salts in a hot air oven at about 90° C. to 100° C. for one hour. The ordinary copper still oven is superior to a flame heated oven and more convenient. The dried paper containing the bile acids is carefully folded, or better still cut into small strips, and inserted into a paper extraction thimble (Whatman thimbles, 22 by 80 mm.). The salts are then subjected to steady extraction with about 25 c.c. of absolute alcohol for sixty minutes, using the modified Soxhlet extraction apparatus (see Fig. 2) recommended by Myers[8] for the estimation of cholesterol in blood. An electric hot plate is used for heating and

great care must be taken not to char the solution by excessive heat. The alcoholic extract is then removed, cooled, and transferred to a 50 c.c. measuring flask, rinsing out the extraction flask with small quantities of absolute alcohol and transfer the washings to the graduated flask, until the volume reaches about 35 to 40 c.c. An alternative but not so reliable method is to place the filter paper containing the bile acids into one of the large boiling tubes and extract with about 5 c.c. portions of absolute alcohol brought to boiling point each time by immersion of the tube in the boiling water-bath. The boiling of the alcohol should be continued for two or three minutes after ebullition. The alcoholic extract is then cooled and filtered through a small 7 cm. paper into the 50 c.c. flask to remove traces of ammonium sulphate. Extraction can be discontinued when 30 to 35 c.c. of alcohol have been used. Two cubic centimeters of a saturated solution of barium hydroxide is then added, and after the addition of a few porous plate chips to prevent bumping, the flask is placed in the boiling water-bath, and the alcohol gently boiled for about three minutes when the total volume will be reduced by about one-tenth. The flask is then removed and thoroughly cooled in running water after which the volume is made up to the mark with absolute alcohol. It might be mentioned that this barium hydroxide treatment does not remove any bile salts from the alcoholic extract. Quantitative figures show an absolute recovery of bile salts.

It is essential to cool the alcoholic extract thoroughly after boiling with the barium hydroxide. If convenient the solution should be allowed to stand from one to two hours so as to allow the baryta treatment to remove the last traces of urinary pigment. A quicker process is to stand the flask in the ice chest for about one hour, or until the temperature of the mixture is round about 5° C. A convenient freezing mixture is ice and dilute sulphuric acid which gives a temperature of about –10° C. (Approximately 50 gm. ice and 60 c.c. of 25 per cent H_2SO_4.) Another good mixture is made as follows: 10 gm. of ammonium nitrate, 10 gm. of sodium carbonate, and 10 c.c. of water. The temperature reached is about 0° C.

After mixing well, filter through a 9 cm. paper that has been previously dampened with a few drops of alcohol. Slight turbidity of the filtrate can be safely overlooked as this does not interfere with the subsequent determination.

Forty-five cubic centimeters of filtrate are measured into one of the boiling tubes, and after the addition of a few silica chips, the alcohol is evaporated to dryness on the boiling water-bath. If the boiling tube is unable to hold 45 c.c. filtrate, first evaporate 25 c.c. of extract almost to dryness and then add the other 20 c.c. of filtrate together with another silica chip to prevent bumping. Keep the water temperature of the bath from about 85° to 90° C. at the beginning of the evaporation and raise the temperature to boiling as the alcoholic solution slowly reduces in volume.

It may occasionally be found that owing to the presence of water which was introduced with the saturated barium hydroxide solution, the last traces of alcohol cannot be removed. The addition of a further 10 c.c. of alcohol will make it possible to evaporate the extract to complete dryness.

It is usual to employ two standards for the urinary process, that is, 0.20 and 0.50 c.c. of the 0.5 per cent alcoholic solution of sodium tauroglycocholate. The alcohol is removed by evaporation on the boiling water-bath.

The color production is now performed on the dried residues. One cubic centimeter of 1 per cent levulose is added to each of the standards and to the unknown residue, and after agitating each tube to break up the precipitate and facilitate solution, 9 c.c. of pure concentrated A.R. hydrochloric acid is then added to all three tubes. After mixing thoroughly, the tubes are securely stoppered and placed in a water-bath for thirty minutes at 38° C. Occasional agitation of the tubes is recommended to ensure an even emulsion and production of color. If bile salts are present a reddish purple color slowly develops which is characterized by a beautiful greenish blue fluorescence. After thirty minutes when the colors have reached a maximum depth, the tubes are removed from the water-bath and cooled in running water. The unknown solution is filtered through a 9 cm. paper, and if the standards are cloudy (and this is always experienced with the stronger standards) they also must be filtered. Select the standard that is nearest the unknown solution by naked eye observation, and then compare in the colorimeter immediately, as fading occurs on standing. As a supplement to the method introduced above, a technic based upon the color production from phosphoric acid and levulose has been utilized. Exactly the same technic of color production is adapted as in the former method, namely, 1 c.c. of 1 per cent levulose and 9 c.c. of pure concentrated A.R. phosphoric acid, but heating is continued for one hour at 38° C. This latter technic, although not so delicate as the hydrochloric acid method somewhat compensates for this drawback, in that the color production is quite stable and also develops to double or even treble the intensity of the original color on standing twenty-four hours. The precipitate in the unknown solution is slightly more difficult to remove with the phosphoric acid method and requires filtration through an asbestos mat in a Gooch crucible (see Fig. 1). A test tube is placed in the pressure flask to collect the filtrate. This may have to be refiltered before it is absolutely clear for colorimetric comparison. The hydrochloric acid treatment will, however, be found to be the more convenient for rapidity of strong color production.

For the experimental work portions of the alcoholic sodium tauroglycocholate of known concentration were measured into the boiling tubes and evaporated to dryness on the boiling water-bath. After the addition of heavily pigmented urines, rich in chromogens and uroerythrin, pure bilirubin was also added in large quantities. The urines were then subjected to the method given above. The results are recorded in Table I and shows the method to be strictly quantitative.

In cases when the estimation of bile salts in test meal (duodenal fluids) samples is necessary, or for the estimation of bile salts in gallbladder bile, the urine technic can be applied after suitable dilution of the specimens, although a direct method of extraction with alcohol is quicker.

Lyon[9] has claimed that it is possible to obtain fractions after the administration of magnesium sulphate which corresponds to the bile, A from the bile

TABLE I

OBSERVED COLORIMETRIC FINDINGS OF STANDARD SOLUTION OF VARYING CONCENTRATIONS OF BILE ACID ADDED TO URINE, AND ALSO THE RECOVERY OF THE NATURAL BILE ACIDS FROM ICTERIC URINES

SERIES	OBSERVATIONS ON URINE EXAMINED	HAY'S TEST	TYPE OF REACTION	SOD. TAURO-GLYCO-CHOLATE ADDED IN MG.	RECOVERED VALUES IN MG.	CORRECTION FOR MG. PER CENT	
						ADDED	RECOVERED
1	Pure solid bilirubin added to the extent of 20 units on V. den Bergh's scale	--	--	20.0	19.0	80.0	76.0
2	Urine contained heavy pigments, with pure bilirubin added to extent of 25 units	--	--	1.25	1.20	5.0	4.8
3	Heavy pigmented urine, added bilirubin 10 units	--	--	1.0	0.80	4.0	3.2
4	Heavily pigmented urine	--	--	0.5	0.52	2.0	2.08
5	Urine rich in urinary pigments. Solid bilirubin added. Bile acids recovered by repeated washings with alcohol and not Soxhlet extraction	--	--	50.0	49.4	200.0	197.6
6	Same as above but using Soxhlet extraction process	--	--	50.0	50.0	200.0	200.0
7	Urine contained very heavy pigments and chromogens. Final filtrate slightly contaminated with pigments	--	--	50.0	54.0	200.0	216.0
8	Urine very heavy in pigments	--	--	10.0	8.6	40.0	34.4
9	Gallbladder bile. 1 in 50 dil. made of pure bile and 25 c.c. (0.45) taken for analysis	--	--	--	17.5	--	70.0 i.e. 3885.0
10	Direct extraction made from the pure bile used above. 0.55 c.c. taken into 50 c.c. alcohol and 40 c.c. extract finally used, viz.: 0.44 c.c.	--	--	--	18.0	--	72.0 i.e. 4086.0
11	Pure bile added to urine. Control made with water	--	--	5.0	5.0	20.0	20.0
12A	Bilirubin in urine — 7 units. Natural bile salts present	Very faintly positive	Direct coloration in the cold on addition of color producing reagents, which faded slightly on heating at 35° C.	--	4.0	--	16.0
13	Urine contained large amount of bile pigment (15 units)	Very faint trace	Immediate color development which developed still more on heating	--	1.1	--	4.4

TABLE I—CONT'D

SERIES	OBSERVATIONS ON URINE EXAMINED	HAY'S TEST	TYPE OF REACTION	SOD. TAURO-GLYCO-CHOLATE ADDED IN MG.	RECOVERED VALUES IN MG.	CORRECTION FOR MG. PER CENT	
						ADDED	RECOVERED
14	Urine contained fair amount of urobilinogen (9.3 units). No bile pigments. 50 c.c. urine used	Markedly positive.	Color developed immediately and faded very slightly on heating	--	2.5	--	5.0
15B	Bilirubin in urine 12 units	Very faint trace	No development of color in the cold, but gradual development of color on heating at 35° C. This is the same as for all body fluids other than urine, and also for the synthetic bile salts	--	2.4	--	9.5
16	Repeated as above but using the H_3PO_4 technic	--	--	--	2.3	--	9.3
17	Urine contained large amount of uroerythrin	Trace present	No immediate color but gradual development on heating in water-bath	--	0.95	--	1.9
18	Repeated but with uroerythrin first removed	--	--	--	0.95	--	1.9
19	Urine contained bilirubin, 14 units. 50 c.c. used	Very faint trace	Immediate color in the cold which faded slightly on heating	--	0.80	--	1.6
20	Urine contained 10 units of bilirubin	Faint trace	Immediate color in the cold which entirely faded on heating	--	--	--	--
21	No urobilin or bile pigments. Urine had a slight icteric tint	Very strongly positive	Absolutely negative	--	--	--	--
22C	Large amount of bilirubin present in urine, 17 units	Trace present	Direct color with complete fading on incubation	--	--	--	--
23	Urine containing urobilinogen and urobilin with trace of uroerythrin and bilirubin	Trace present	Very heavy direct coloration in the cold with complete fading on heating	--	--	--	--
24	Urine very heavily pigmented but with only a trace of bile pigments present	Strongly positive	Negative	--	--	--	--
25	Urine heavily pigmented with large amount of bilirubin	Faint trace	No immediate color in cold but gradual development of color on heating	--	0.50	--	2.0
26D	Catarrhal jaundice. Urine contained 5 units of bilirubin. Trace of uroerythrin. 50 c.c. used	Strongly positive	No immediate color in cold but gradual development of color on incubation	--	9.3	--	18.6

TABLE I—CONT'D

SERIES	OBSERVATIONS ON URINE EXAMINED	HAY'S TEST	TYPE OF REACTION	SOD. TAUROGLYCOCHOLATE ADDED IN MG.	RECOVERED VALUES IN MG.	CORRECTION FOR MG. PER CENT	
						ADDED	RECOVERED
27	Test meal sample (bile stained). 10 c.c. used	Strongly positive	--	--	10.0	--	100.0
28	Urine very heavily pigmented containing moderate amount of bilirubin (11 units)	Strongly positive	No coloration in the cold but gradual development on heating	--	3.75	--	15.0
29	Same as above but using the H_2PO_4 method	--	--	--	3.83	--	15.3
30	Chronic interstitial nephritis. A second estimation was performed allowing the color to develop in the cold for 10 minutes and comparing colorimetrically. 50 c.c. of urine taken	Faintly positive	Strong immediate coloration in the cold with fading on heating at 35° C.	--	2.30	--	4.60
31	Heavily pigmented urine containing fair amount of urobilinogen and large amount of bilirubin, 12 units	Very strongly positive	No coloration in the cold, but strong development of color on incubation	--	5.3	--	21.2
32	Acute nephritis. Second estimation without incubation, but allowing the color to develop in the cold for ten minutes and comparing colorimetrically. 50 c.c. of urine used	Markedly positive	Very strong immediate coloration in the cold with fading on incubation	--	11.7	--	23.4
33	Carcinoma of liver. Urine contained no bile pigments but a large amount of uroerythrin. 50 c.c. of urine used	Faint trace	An immediate coloration in the cold with slight fading on incubation	--	2.0	--	4.0
34	Same as above but using the H_2PO_4 method	--	Immediate coloration in the cold but no further development on heating	--	2.4	--	4.8

ducts, B from the gallbladder, and C from the liver, but other workers do not agree that the bile from these three sources can be fractionated successfully. The most important part of the analysis is the bilirubin and bile salts fractions.

Chiray and Lebon state that with bilirubin and bile salts there may be little or nil in obstructive jaundice, but an increase in familial jaundice, chronic splenomegalic icterus, hemolytic jaundice, paroxysmal hemoglobinuria and pernicious anemia. In catarrhal jaundice the concentration of bilirubin and bile salts may be normal, but the total output low. There may be an increase or decrease of bile salts in parallel with bilirubin, or there may be "dissociation" as in hemolytic jaundice (see Chiray and Lebon's book, *Le Tubage Duodenal*, 1924).

In the above book Carnot and Mauban describe a technic employing Hay's test[10] and have thereby obtained an approximate measurement. The new process is introduced to give a strictly quantitative method.

With test meal samples dilute 2 or 5 c.c., depending upon the icteric tint of the specimen, to 25 c.c. with water and proceed as for the urine process. For the direct extraction add the above amount of gastric juice into about 30 c.c. of alcohol, add a few porous chips and heat on the water-bath for two or three minutes. After cooling, dilute to 50 c.c. with alcohol. Filter through a 9 cm. paper. Forty-five cubic centimeters of filtrate are pipetted into a 100 c.c. capacity Erlenmeyer flask, 3 c.c. of the saturated solution of barium hydroxide are added plus a few porous chips, and the mixture is gently boiled on the boiling water-bath for about three minutes after which it is thoroughly cooled in running water. Now transfer it to a small measuring cylinder and make up the total volume of the mixture to 45 c.c. with alcoholic washings from the Erlenmeyer flask. Filter through a 9 cm. paper. Forty cubic centimeters of alcoholic filtrate are pipetted into a boiling tube, and after the addition of a few silica chips, evaporated to dryness on the water-bath. The color production is exactly the same as for the urinary process. The standard usually employed is 1 c.c. of the 0.5 per cent standard solution.

For Gallbladder Bile.—Twenty-five cubic centimeters of 1 in 100 dilution of gallbladder bile is treated exactly as for the urinary process. With the direct extraction 0.25 c.c. of pure bile is added by means of a blood pipette to about 30 c.c. of alcohol, after which the test meal method for the direct extraction of bile acids is followed. Two cubic centimeters of the 0.5 per cent solution is used as standard.

The strength of the color production is reported in terms of milligrams of tauroglycocholate for each 100 c.c. of urine, calculated by the formula:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{\text{Strength of standard (1 or 2.5 mg.)}}{\text{Amount of urine used (25 c.c.)}}$$

$$\times \text{ Fraction used } \left(\frac{50}{45}\right) \times 100 = \text{mg. of bile salts per 100 c.c. of urine.}$$

Interpretation of Results.—An interesting fact emerges from this reaction, namely, the difference in color reaction from urinary extracts may be due to two distinct types of bile salts, Types A and B.

With Type A, the reaction is the usual response always obtained from all other body fluids, viz., no production of color in the cold, but a gradual development of color reaching a maximum in thirty minutes on warming in the water-bath at 38° C.

With Type B, the production of color begins immediately or after a few minutes in the cold, depending upon the amount of bile salts present. This coloration either develops slowly or fades entirely on heating. It is suggested that the B type of bile salts are exceedingly unstable with the result that the color development fades rapidly on heating. When, however, there is a combination of these two types of bile salts it will be observed that the color pro-

duced in the cold appears to develop on heating. The explanation may be that the B type of bile salts is stabilized by the presence of the A type, or again, on heating, the color developed from the A type of bile salts entirely obscures the fading of the color from the B type.

To overcome this difficulty the color that develops in the cold should be allowed to stand a further fifteen minutes, and after filtration compared in the colorimeter against a suitable standard prepared by heating at 38° C. in the usual way. The other process can be used by which the color can be stabilized, namely, the utilization of the phosphoric acid technic. This method will have to be adopted for all specimens of urine, as, of course, it is impossible to know when a direct production will be obtained, or again, if this coloration will develop or fade on incubation. But as already stated the phosphoric acid technic is not so convenient as the hydrochloric acid treatment, in that with the majority of urines containing small amounts of bile salts, the specimens must be allowed to stand for twenty-four hours until sufficient color has developed for colorimetric comparison.

The new reaction also disagrees with Hay's test for bile salts. This is to be expected as Hay's test depends on a lowered surface tension of the urine, and therefore is not specific for bile acids. It has been observed that substances lowering the surface tension may occur in certain cases of nephritis. Urines examined from interstitial and acute nephritis and giving a positive Hay's test show the characteristic reaction of the B type of bile salts.

Therefore, it must be concluded, by the new reaction proving these so-called "substances" to be bile salts, that the nephritic toxemias cause derangement of hepatic function. Recapitulating, it may be said that the B type of bile salts do not react to give the characteristic hydrochloric acid levulose-colored compound, and also, the majority of these bile salts remain in the filtrate from the urine and are therefore not precipitated with hydrochloric acid and ammonium sulphate. It is quite possible that the latter part of the theory given below explains these interesting findings.

The difference in the color reaction may depend either upon some slight variation in the bile salt molecule, or upon bile salts being present in some sort of association with cholesterol or a urinary constituent, which might well modify their physical properties.

Another interesting fact is that the cholesterol in the gallbladder is kept in solution as an emulsoid colloid as a result of the solvent action of bile salts. With deranged hepatic function the close association of cholesterol and bile acids makes the above theory feasible.

These are the observed facts, and it is idle to speculate as to their meaning until more is known of the physiology of the subject.

For Blood.—Into a 50 c.c. measuring flask containing about 30 c.c. of absolute alcohol, add 5 c.c. of whole blood, serum, or plasma. Immerse the flask in the boiling water-bath with constant agitation for two or three minutes, cool, and dilute to the 50 c.c. mark with alcohol. The protein precipitate should be allowed to separate roughly before filtering as this saves considerable time with filtration. The supernatant fluid is then filtered through a 9 cm. paper.

Transfer 40 c.c. of filtrate to a 100 c.c. Erlenmeyer flask, add 1 c.c. of saturated solution of barium hydroxide, and after adding a few small porous chips place in the boiling water-bath for about two minutes. The mixture is thoroughly cooled, transferred to a measuring cylinder and the volume made up to 40 c.c. with alcoholic washings from the Erlenmeyer flask. After filtering through a 9 cm. paper, 35 c.c. filtrate are pipetted into a boiling tube, a few silica chips are added, and the alcoholic extract is taken to dryness on the boiling water-bath. If frothing takes place toward the end of the evaporation this can be prevented by the addition of one or two drops of caprylic alcohol. The standard is prepared by taking 5 c.c. of a 0.005 per cent solution of sodium tauroglycocholate (1 c.c. of 0.5 per cent solution diluted to 100 c.c. with absolute alcohol) to dryness on the water-bath.

The colors are produced in a volume of 2.5 c.c., i.e., 0.25 c.c. of 1 per cent levulose and 2.25 c.c. of strong concentrated hydrochloric acid. The "unknown" solution is too small to be filtered and must be transferred to a centrifuge tube, and after securely stoppering the tube, centrifuge at high speed. Although by centrifuging the supernatant fluid never becomes absolutely clear this does not annul the delicacy of the reddish purple coloration. The unknown solution is then compared against the standard color in a similar tube by naked eye observation. If the unknown color is stronger than the standard solution it must be diluted with pure concentrated A.R. hydrochloric acid until the colors match. On the other hand if the standard solution is the stronger color, it must be diluted likewise with the hydrochloric acid. The volumes of both the solutions are now taken. This process must be called simply "an estimation," and not a quantitative determination.

$$\frac{\text{Volume in centrifuge tube of unknown solution}}{\text{Volume in centrifuge tube of standard solution}} \times \frac{\text{Strength of standard in mg.}}{\text{Amount of blood used}}$$

$$\frac{50}{35} \times 100 \text{ equals mg. of bile acids per 100 c.c. of blood.}$$

There can be little doubt that a mechanism for the manufacture of bile salts exists in the living body. In the presence of a biliary fistula such salts are continually excreted which would be impossible but for their endogenous formation. Moreover, those bile salts excreted into the duodenum are reabsorbed lower down in the intestinal tract, for none can be detected in the feces. They must therefore be circulating in the portal circulation and unless picked up quantitatively by the liver must circulate in the general blood stream and should therefore be detectable. Although some of the workers quoted earlier were unable to demonstrate the presence of bile salts in normal blood, it is by no means justifiable to conclude that they are absent. The results recorded in Table II clearly show that small quantities of bile salts added to normal blood in vitro could not be recovered. In fact on the average 5 mg. of bile salts were always lost, whatever the quantity added to 100 c.c. of blood. Lesser quantities were completely lost. It is assumed that they were carried down with the protein precipitate on the addition of alcohol, though in what form it is difficult to say. It may be possible that the bile salts are in combination with one of the constituents of the protein precipitate in which form

TABLE II

SHOWING RECOVERY OF BILE ACIDS ADDED TO BLOOD AND FINDINGS IN VARIOUS CONDITIONS

SERIES	OBSERVATIONS ON BLOOD EXAMINED	SOD. TAURO-GLYCOCHOLATE ADDED IN MG.	RECOVERED VALUES IN MG.	CORRECTION FOR MG. PER CENT	
				ADDED	RECOVERED
1	0.20 mg. of bile salts added to 5 c.c. blood plasma. Color produced in volume of 5 c.c.	0.20	0	4.0	0
2	0.25 mg. of bile salts added to 5 c.c. serum. Color produced in 2.5 c.c. volume	0.25	0	5.0	0
3	0.25 mg. of bile salts added to 5 c.c. blood plasma. The alcoholic extract was taken direct to dryness and not subjected to the barium hydroxide treatment. Color produced in volume of 2.5 c.c.	0.25	0	5.0	0
4	0.25 mg. of bile salts added to 5 c.c. whole blood, color produced in volume of 2.5 c.c.	0.25	0	5.0	0
5	1 mg. of bile salts added to 5 c.c. of whole blood; color in 7 c.c.	1.00	0.75	20.0	15.0
6	0.5 mg. of bile salts added to 5 c.c. whole blood; color in 5 c.c.	0.50	0.30	10.0	6.0
7	5 mg. of bile salts added to 5 c.c. whole blood	5.0	4.5	100.0	90.0
8	7 c.c. of normal plasma taken for analysis	--	--	--	0
9	10 c.c. of normal serum used	--	--	--	0
10	10 c.c. whole blood	--	--	--	0
11	20 c.c. whole blood	--	--	--	0
12	10 c.c. whole blood treated as for the Aldrich and Bledsoe process	--	--	--	0
13	Blood giving delayed Van den Bergh reaction; indirect reaction 4.7 units. 5 c.c. of serum used	--	--	--	0
14	Pernicious anemia. Van den Bergh reaction, indirect only, giving 18 units	--	--	--	7.0
15	Splenic anemia. 4 c.c. blood taken, and color produced direct from alcoholic extract	--	--	--	0
16A	Blood gave indirect Van den Bergh reaction. 5 c.c. of whole blood used	--	--	--	9.0
17B	A strong immediate Van den Bergh reaction. 4 c.c. of serum used	--	--	--	23.0
18C	Very strong immediate direct Van den Bergh reaction. 50 units of bilirubin	--	--	--	47.0
19	Blood giving positive indirect Van den Bergh test of 5 units	--	--	--	0
20D	Catarrhal jaundice. Immediate direct reaction. 7 units of bilirubin present	--	--	--	0
21	Jaundice. Biphasic Van den Bergh reaction. 11 units of bile	--	--	--	0

they actually circulate in the blood stream. Or again, the blood proteins may possess the property of "adsorbing" a certain quantity of the bile salts after which these cannot be redissolved by the absolute alcohol. This seems most likely as the ratio between bile salts and blood protein per 5 c.c. of blood is 0.25 and 350.0 mg. respectively. The amount of bile salts lost varies slightly with the total protein content of the blood.

The present investigation proves definitely that the blood is able to conceal a small quantity of bile salts so that they escape detection. Those workers, therefore, who found bile salts absent in normal blood, actually found only their absence above a certain concentration, i.e., 5 mg. per 100 c.c. of blood. Whatever the amount of blood used the amount of bile salts carried down by the protein precipitate remains proportionally constant, viz., 5 mg. per 100 c.c. of blood. Furthermore, with methods employing a latter stage in the alcoholic extract, such as the addition of norite to remove coloring matter, a large quantity of bile salts are also removed. No matter what method is used to isolate the bile salts (and extraction with alcohol is by far the most successful), a small amount of bile acids is lost in the albuminous precipitate, which again varies considerably with the protein precipitant.

With a view to recovering the bile salts from the protein precipitates various methods were tried, such as, first the drying of blood on filter paper and extracting with absolute alcohol in the modified Soxhlet apparatus for one or two hours. Second, blood protein was removed by precipitation with absolute alcohol and then collected and subjected to steady extraction with absolute alcohol in the Soxhlet extraction apparatus from two to three hours. The results were negative in those samples to which bile salts had been added to the extent of 5 mg. per 100 c.c. of blood, as well as in bloods to which no bile salts were added.

Finally, investigation showed that even if the alcoholic extracts from very pale serums containing added bile salts (0.25 mg. per c.c. of blood) were taken direct to dryness and not subjected to the barium hydroxide treatment (there being practically no coloring matter to remove) absolute negative results are obtained. In the above experiments the color production was performed in a total volume of 2.5 c.c., i.e., 0.25 c.c. of levulose and 2.25 c.c. of concentrated hydrochloric acid. The technic employed for the blood process is more useful as a qualitative than a quantitative method, first, because a reaction is only obtained when the amount of bile salts in the blood rises above 5 mg. per 100 c.c., and second, because a measurable color reaction cannot be obtained with quantities of blood less than 5 c.c. However, large quantities of blood can be used if available. An excess of bile acids can easily be demonstrated in the blood and the method is therefore very satisfactory.

With latent jaundice the test is rather unsatisfactory due presumably to the "holding back" of the bile acids in the protein precipitate. Here the estimation of bilirubin is superior. Further work, however, may give interesting results in connection with dissociated jaundice, i.e., independent retention or excretion of bile pigments and bile salts.

Hemolytic jaundice is certainly a dissociated condition, since the bile salts are unaffected. Retention of bile salts alone has been observed in the later stages of catarrhal jaundice.

For Feces.—Weigh out 2 gm. of wet feces with the aid of a glass rod, into one of the thick-walled boiling tubes that has been previously graduated at 50 c.c. If the feces is exceptionally liquid 3 gm. can be used. Add about 30 c.c. of alcohol and break up as much of the solid material as possible by stirring

well. After adding a few porous chips immerse the tube in the boiling water-bath for two to three minutes, cool, and dilute to 50 c.c. with absolute alcohol. Allow the precipitate roughly to settle and then carefully pour off supernatant extract on to a 9 cm. paper. Transfer 40 to 45 c.c. of filtrate to a 100 c.c. capacity Erlenmeyer flask, add 2 c.c. of the saturated solution barium hydroxide plus a few porous chips. Heat on the boiling water-bath for about three minutes or until the mixture has concentrated about one-tenth. After thoroughly cooling the solution in running water, make up to the original volume (viz. 40 or 45 c.c.). Filter through a 9 cm. paper. Pipette 35 or 40 c.c. of filtrate into a boiling tube together with a few silica chips, and evaporate to dryness on the water-bath.

It will be noticed that the fecal residue contains much more pigment than either the urine or the blood extracts, but this does not annul the subsequent color production as the pigments and fats are rendered insoluble by the hydrochloric acid introduced in the color production stage of the method. The

TABLE III

SHOWING EFFECT OF BILE ACIDS ADDED TO FECES AND THEIR SUBSEQUENT RECOVERY

SERIES	OBSERVATIONS ON FECES EXAMINED	SOD. TAUROGLYCOCHOLATE ADDED IN MG.	RECOVERED VALUES IN MG.	CORRECTION FOR MG. PER CENT IN WET FECES		CORRECTED FOR DRY WEIGHT	
				THEORETICAL	FOUND	THEORETICAL	FOUND
1	Normal feces. Two grams wet feces used. Solid material 30.00 per cent	5.0	4.4	250.0	220.0	833.0	733.0
2	Normal feces. Three grams wet feces used. Solid percentage 14 30	5.0	4.7	166.6	156.6	1650.0	1095.0
3	Gross diarrhea. Three grams wet feces used. Solid percentage 8.33. Bilirubin present to extent of 93 units per 100 gm. of dry feces	--	2.1	--	70.0	--	840.0

insoluble fats and pigment are then removed by filtration. Two standards should be prepared 0.2 and 0.5 of the 0.5 per cent standard solution. The color production is the same as for the urinary process.

Calculation:

$$\frac{\text{Standard reading}}{\text{Unknown reading}} \times \frac{\text{Strength of standard in mg.}}{\text{Amount of feces taken}} \times \frac{50}{35} \text{ (or the amount of alcoholic extract finally taken to dryness)} \times 100 = \text{mg. of sodium tauroglycocholate per 100 gm. of wet feces.}$$

To obtain the solid percentage of the feces examined, weigh out exactly 1 gm. of wet feces into a small porcelain dish and dry by means of a boiling water-bath and vacuum desiccator. The weight of the remaining residue multiplied by 100 gives the solid material expressed in percentage. Now if the amount of bile salts present in 100 gm. of wet feces is multiplied by:

$$\frac{100}{\text{Solid percentage of feces}},$$

the percentage of bile salts per 100 gm. of dried feces will be ascertained.

The results obtained from the feces process are highly satisfactory, considering the extremely heavy pigmentation and nature of the material. The method is not of value as a test for hepatic function, but is useful as regards an excessive secretion of bile into the intestine. In gross diarrhea the feces excretion may contain bile acids. The process is introduced for those interested in this type of test.

SUMMARY AND CONCLUSIONS

1. A new colorimetric method is described which is regarded as more satisfactory for the clinical investigation of the bile salt content of body fluids, than any other method in existence. The method is entirely satisfactory in every respect as regards body fluids with the single exception of the urinary process, where limitations imposed upon the technic owing to the presence of both types of bile salts interfere with the reaction, though despite this, very interesting results are yielded. This is not due to any flaw in the method itself but is thought to be due to some chemical or physiochemical difference in the bile salts. It may be mentioned that if the bile salts present in the urine are "characteristic" of those found in other body fluids, they can be precipitated from solution with hydrochloric acid and ammonium sulphate, and the color reaction produced in the usual way, i.e., no production of color in the cold, but gradual development of color on heating in the water-bath. On the other hand the development of color in the cold may be due to bile salts in combination with cholesterol or a urinary substance. In the majority of cases only a very small quantity of the B type of bile salts is precipitated by hydrochloric acid and ammonium sulphate, but again in a few specimens the full quantity can be removed. With the phosphoric acid process the B type of bile salts still produce an immediate coloration in the cold, which does not fade on heating at 38° C. for one hour. The coloration so produced is of a dull red hue and not the characteristic reddish pink color obtained by the hydrochloric acid method. With the A type of bile salts both the hydrochloric acid and phosphoric acid methods give the reddish purple coloration.

2. The method is unable to show evidence of the presence of bile salts in normal blood, nor can it definitely state that bile salts are completely absent, and observations made during the experimental stages of the present method neither proves nor disproves their presence. The important fact noted with the blood analysis is that a certain amount of bile salts are always lost in the protein precipitate. This may be due to the bile salts forming a compound with protein sufficiently stable to resist extraction with alcohol, although it seems most likely that the bile salts are "adsorbed" by the protein precipitate on the addition of alcohol. The amount lost is found to be approximately 5 mg. per 100 c.c. of blood. When in liver diseases the bile acids rise above this amount their presence in the blood can be demonstrated. The method for blood is only "an estimation" and not a quantitative procedure. This is due not only to the amount of bile salts lost in the protein precipitate but also to the large quantity of blood required. With "latent" jaundice the blood process is not of great practical value, but is useful with regard to dissociated jaundice.

3. A method is provided for bile salts in feces, although their presence is of little significance.

4. The reaction has numerous advantages over other tests which have been proposed: (a) Its extreme delicacy; (b) is not given by substances other than bile acids that are present in body fluids; (c) is absolutely satisfactory quantitative method for the determination of biliary acids; (d) is a great improvement on those methods employing the Pettenkofer reaction.

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A SIMPLE AND INEXPENSIVE APPARATUS FOR THE DETERMINATION OF THE METABOLIC RATE IN GUINEA PIGS*

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WE WISH to present the details of an apparatus for the determination of the basal metabolic rate in guinea pigs, not because of any originality connected with its construction, but because of its simplicity and inexpensiveness. Several different types of apparatus were tried, but it required several weeks of experimentation with each to learn that, at least in our experience, the results became more constant as we simplified the apparatus. For animals as small as guinea pigs, we feel quite certain that the "closed system" type of apparatus is more practical than any other. All of the articles of glassware, etc., used in the construction are so simple that they can be found or constructed in almost any laboratory.

Description of Apparatus.—Two large glass bottles with a capacity of 2,300 c.c. to 2,500 c.c., like those used for containers of sulphuric acid, are fitted with rubber stoppers, and a hole about 2 cm. in diameter is bored in the bottom of each (see Fig. 1). One of these bottles (*B*) is fixed to a tripod on a table and the other (*A*) suspended with a cord over a pulley so that it can be lowered or raised. A thermometer is inserted through a hole in the stopper of the stationary bottle (*B*). Two bottles of about 500 c.c. capacity (to be used as absorption bottles) having large openings at the top are fitted with rubber stoppers, and a glass tube inserted through one of the holes in the stopper down to the bottom of each bottle. A small cage of wire screen is constructed and placed in the bottom of each bottle so as to prevent the hole of the tube from blocking with soda lime. A hole about 2 cm. in diameter is bored in the bottom of a 500 c.c. graduated cylinder and by means of a small pulley the cylinder is suspended into a larger glass cylinder measuring 45 cm. in height and 7 cm. in diameter. This serves as a spirometer. This latter cylinder is filled nearly to the top with water. A piece of glass tubing is bent in a narrow U-shaped fashion and anchored on the rim of the outer cylinder of the spirometer. The arm of the U-tube which is placed within the graduate cylinder is made long enough to protrude beyond the water level. The current of gas passing through this tube and out the top of the spirometer or vice versa, insures adequate circulation of the gas within the spirometer. A three-way glass stopcock is inserted between the spirometer and Bottle *B* so that oxygen may be introduced into the spirometer at the beginning of the metabolic test. A bottle with a capacity of about 1,500 c.c. and having a large hole in the top is used as the animal chamber (*F*). This jar is fitted with a rubber stopper

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containing 3 holes. Into one hole is fitted a thermometer; into the other 2 holes glass tubes are inserted. A piece of rubber tubing 15 to 18 cm. long is attached to one of the tubes on the inside surface of the stopper so that the circulation in the animal chamber produced by raising or lowering Bottle *A* will be efficient. Bottle *A* is filled with water and a small amount of copper sulphate introduced. If an antiseptic is not added to the water, the viscosity will be changed by bacterial growth, thus altering the emptying time of the bottle. The absorption bottles are filled two-thirds full with soda lime.* A suitable basin, either glass, galvanized iron or copper, measuring about 45 by 20 by 20 cm. is obtained and filled one-half full with water so that the animal chamber containing the guinea pig can be immersed when a determination is being made. This prevents sudden changes in the temperature of the air within the animal chamber. The walls of this basin should be painted black so that by placing cardboard or paper over the top, the animal is confined in a darkened chamber. This is an important factor in eliminating movement. The animal chamber should be anchored in the water basin and balanced in such a way that movement of the animal can be detected without admitting light to the water-bath.

The stoppers are fitted with glass tubing and the proper connections (see Fig. 1) made with a good grade of rubber tubing with fairly thick walls. The stoppers which need not be removed are sealed tightly with glue and all rubber to glass connection reinforced with wire. The rate of the flow of water from the "lift" Bottle *A* to Bottle *B* is regulated by the distance the "lift" Bottle is raised or lowered from the level of Bottle *B*. It should be regulated so that one bottle empties in exactly two or two and one-half minutes. Bottle *A* may be raised and lowered either by hand or by motor.

The system *must* be air tight. It is tested for leaks by inserting the stopper in the animal chamber, filling the spirometer with air and placing a 100 gm. weight on top of the spirometer. With Bottle *B* empty, the volume of gas in the spirometer is noted and Bottle *A* raised. When empty, it is lowered, and after Bottle *B* is again empty, the volume of gas in the spirometer is noted. If the volume of gas in the spirometer is the same as before the test, there should be no leaks, providing the temperature within the system is unchanged. Until one is convinced that the apparatus does not leak, a further test for leaks should be made after each metabolic determination by placing a weight on the spirometer with the water level in Bottles *A* and *B* stationary. The loss in volume within the spirometer should be no greater than the consumption of oxygen by the animal.

Method of Determination of Metabolic Test.—After immersing the animal chamber containing the guinea pig in the water-bath, the spirometer is filled with oxygen and the gas circulated for four or five minutes by raising and lowering Bottle *A*. After Bottle *B* is completely empty, the menisci in the inner and outer cylinders of the spirometer are adjusted to the same level and the volume of gas in the graduated cylinder, and the time noted. The temperature in

*We use Wilson's soda lime which is so extensively and satisfactorily used in determining the metabolic rate in human beings.

Bottle *B* and the animal chamber is recorded. Bottle *A* is immediately lowered. The distance Bottle *A* is lowered or raised is adjusted so that the bottle will empty and fill in exactly five minutes. A record of the amount of oxygen consumed is thereby obtained every five minutes. It is necessary to continue the determination for at least twenty minutes so that any errors in reading gas volume, temperature, etc., are minimized. It is very important that gas

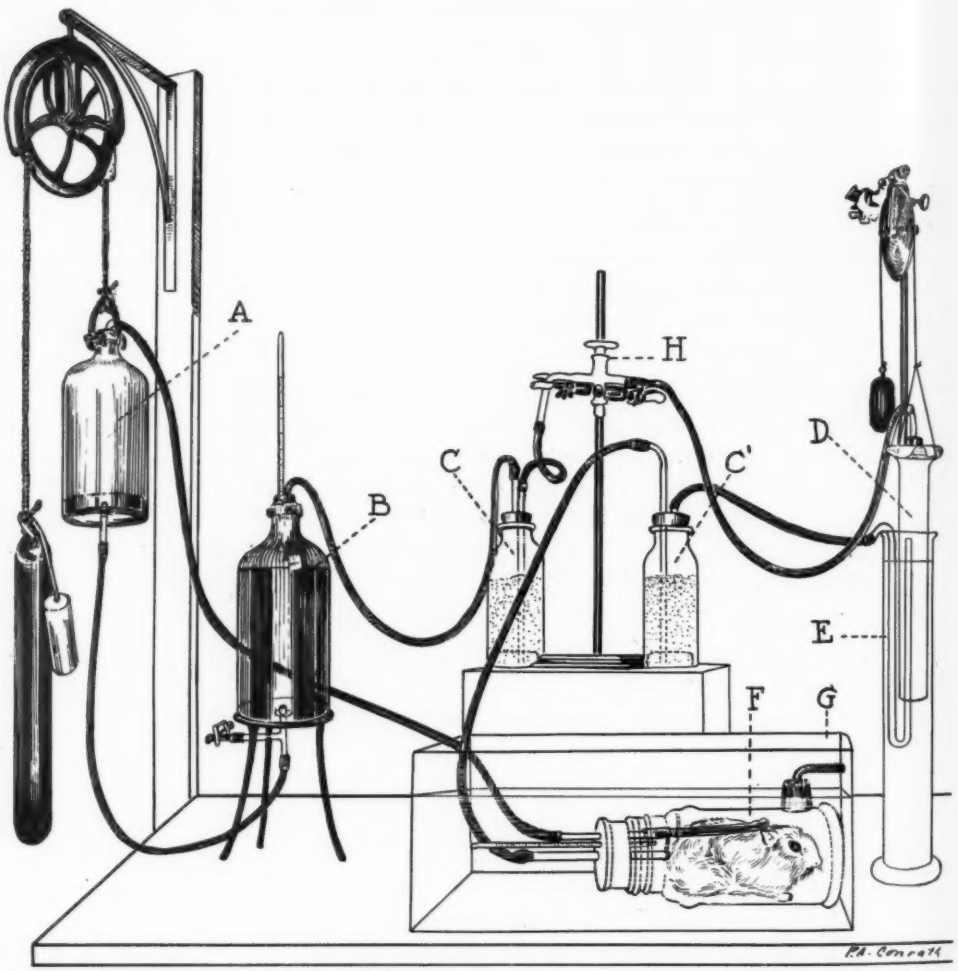

Fig. 1.—*A*, "Lift" bottle. *B*, Stationary bottle. *C*, *C'*, Absorption bottles containing soda lime. *D*, Graduated inner cylinder of spirometer. *E*, Outer cylinder of spirometer. *F*, Animal chamber. *G*, Water-bath. *H*, Three-way stopcock.

volume in the spirometer be recorded only when Bottle *B* is empty. If water is flowing from Bottle *A* to *B* or vice versa, the pressure required to move the water is sufficient to change the volume of gas in the spirometer. By recording the amount of oxygen consumption every five minutes, it is possible to detect errors which are brought about by movement, change in temperature, leaks, etc. At the end of the determination the temperature in the animal chamber and Bottle *B* is again recorded.

Before the volume of gas is reduced to 0° C. and 760 mm. pressure, adjustment must be made for any change in temperature within the system while the determination was being made. To make this correction, it is necessary to know the volume of the various components of the system. For every degree variation in temperature, an addition or subtraction of 0.004 c.c. for each cubic centimeter of gas in the system must be made, depending on whether the temperature rises or falls. It is probably more accurate to calibrate the system. To do this all draughts are eliminated from the room, and the temperature of the air within the bottles of the apparatus as well as the room must have been constant for at least thirty minutes before the calibration is made. The temperature of the water-bath containing the animal chamber is then raised 1° or 2° by the addition of hot water to the water-bath. To be accurate, an object approximately the size of a guinea pig, should be placed in the animal chamber before this calibration is made. The change in volume of air within the system for each degree change of temperature within the animal chamber is easily determined. In our apparatus 1° rise in temperature within the animal chamber produced an increase of 5.9 c.c. in the volume of the system as recorded by the spirometer. With the temperature of the animal chamber constant, a current of heated air is turned on Bottles *A* and *B* until the temperature has reached a constant temperature. With our apparatus a rise of 1° in the temperature of Bottle *B* caused an increase of 13.6 c.c. in the volume of the system as recorded by the spirometer.

Sample determination: Wt. of guinea pig 450 gm. Bar. Pr. 736 mm. Hours' starvation eighteen.

SPIROMETER READING	TIME		TEMPERATURE	
		ANIMAL CHAMBER	BEFORE	AFTER
236	11:50		27.6°	27.3° (drop of 0.3°)
206	11:55			
176	12:00	Bottle *B*	27.3°	27.2° (drop of 0.1°)
145	12:05			
114	12:10			
122 c.c.				

Oxygen consumption in 20 minutes = 122 c.c.
Correction for drop in temperature in animal chamber $-0.3 \times 5.9 = 1.77$ c.c.
Correction for drop in temperature in Bottle *B*. $0.1 \times 13.6 = 1.36$ c.c.
Total 3.13 c.c.
Oxygen consumption in 20 minutes (corrected) = 122 − 3 = 119 c.c.
Oxygen consumption in 1 hour = 357; at 0° and 760 mm. = 304.5 c.c.

$$\text{Oxygen consumption per kilo per hour} = \frac{304.5}{450} = 676 \text{ c.c.}$$

As long as the weight of the animal remains approximately the same, we find it more convenient and just as accurate to record the oxygen consumption in cubic centimeters per kilo per hour, instead of cubic centimeters or calories per square meter per hour. We feel that this method is just as accurate, especially when one considers that the shape of individual guinea pigs does not vary as it does in human beings. We do find it more accurate, however, to establish an average metabolic rate for each animal by making determinations every other day for several days, and to use that average as the metabolic

rate for that particular animal, since it is true that the metabolic rate of one animal may vary 10 or 12 per cent (see Table I) from that of another.

We have listed below a series of 10 animals upon which determinations were performed during the summer months and another series of 10 with determinations made during winter months. The figures represent an average of from 4 to 8 determinations upon that particular animal. The animals listed below represent consecutive experiments, except that out of each series of 10, one animal was discarded because of exceptionally high rate caused by uncontrollable motion of the animal.

TABLE I

METABOLIC RATE c.c. per kilo per hour	
10 ANIMALS IN SUMMER	10 ANIMALS IN WINTER
640	706
675	714
663	702
635	715
634	635
666	640
600	626
581	610
621	758
635	714
Average 635 c.c. Variation of −6 to +8 per cent from the average	Average 682 c.c. Variation of −9.5 to +11 per cent from the average

From the figures in the above table it will be observed that the average metabolic rate was approximately 7 per cent higher in winter than in summer. The individual variation was slightly greater in winter than in summer.

No attempt was made to weigh the various foods given the animals, but it is important that the diet is not deficient in certain necessary constituents. In our experience, feeding lettuce, carrots, oats, and alfalfa, would maintain a healthy normal guinea pig.

It is essential that animals of relatively the same age and weight be used. We have had the most consistent results with guinea pigs weighing between 400 and 480 gm. Any sudden loss of weight due to starvation or illness will lead to erroneous results (elevation of the metabolic rate).

We have made many determinations using a preliminary subcutaneous injection of 7 or 8 mg. sodium amytal twenty minutes before the test, and as far as we could determine obtained accurate results. The average oxygen consumption in consecutive animals receiving sodium amytal was 608 c.c. per kilo per hour, or 12 per cent lower than a series conducted at the same time without amytal. The individual variation in metabolic rate was −5 per cent and +8.5 per cent from the average, or very slightly less than the variation in animals not receiving amytal. The dose of 7 or 8 mg. for an animal of about 450 gm. was sufficient to make it close its eyes and lower its head as if asleep, but not enough to make it lie down. It recovers from the effects of this dose very rapidly but not before the determination is completed.

PRECAUTIONS

1. The system *must* be absolutely air tight. It should be tested frequently, if not after each determination by putting a weight on top of the spirometer and observing the gas volume within the cylinder.

2. At first thought, motion of the animals would appear to be a serious source of error. With the exception of an occasional animal, which must be discarded, this error can be eliminated, even though a guinea pig cannot be trained as can dogs or some other animals. Usually, darkening the chamber while the test is being made is of great advantage. If this is done, the animal chamber must be balanced in such a way so that motion can be detected. By recording the rate of consumption every five minutes, however, any movement will be quickly detected by the increased oxygen consumption. The animals must be starved at least fourteen hours, before each determination, but if starved for more than eighteen, they are apt to be restless on account of hunger. Guinea pigs do not tolerate cold quarters. If a metabolic determination is made while the animal is cold, it will, as expected, be elevated 15 to 25 per cent. It is therefore essential that the temperature of their quarters as well as the room where the determinations are made, be kept as warm as 24° C. and that the animal chamber also be kept warm (preferably 25° to 28° C.) while tests are being made. The apparatus must be protected from draughts of air. Although one can correct for changes in temperature within the system, this change will lead to less error if it is reduced to a minimum.

3. When gas volumes are recorded, the spirometer must be adjusted so that the two water menisci are on the same plane, and the reading must be made a few seconds after Bottle *B* has emptied.

4. Before each determination, the spirometer must be emptied and filled with oxygen, not air. If air is used, the depletion of oxygen within the system may lead to an enormous error (decrease in consumption as measured by the spirometer).

SUMMARY

We have described a simple apparatus, easily and cheaply constructed, which in our hands has yielded constant and presumably accurate results in determining the metabolic rate in small animals. We have used it only on guinea pigs, but see no reason why it could not be used for rats, especially if the sizes of the various chambers were reduced. By using proper precautions and discarding an occasional animal, movement can be entirely eliminated as a serious source of error. No claims for originality in construction of the apparatus are made.

A COMPARISON OF THE WASSERMANN AND KAHN REACTIONS UPON SPINAL FLUID IN TREATED AND UNTREATED CASES OF PARESIS*

DE LESTER SACKETT, B.S., AND ERIC ESELIUS, B.S., ELGIN, ILL.

A LARGE number of articles have appeared in the literature comparing the results of the Wassermann and Kahn reactions since Kahn introduced his test in 1922.[1] The Kahn reaction has been compared with many Wassermann technics, and at the present time it is generally accepted that there is a very close agreement between the results of the two tests upon blood serum.

Although we cannot agree with those individuals who, for various reasons, recommend that the Kahn can completely replace the Wassermann reaction upon blood serum as a routine procedure, we do believe that the Wassermann tests should likewise not be run independently. It is our opinion that the greatest value of any precipitation reaction is that it serves as a necessary check to the complement fixation reactions. All complement fixation reactions should be controlled by a recognized precipitation test, and vice versa, and never by a technic based upon the same underlying principles.

The Kahn reaction upon spinal fluid is also of value in the serology of syphilis, but has not been accorded the enthusiastic approval received by the same test upon the blood serum. Although many articles have been published containing a series of parallel Wassermann and Kahn reactions, which show a close agreement between the two tests, articles have appeared indicating that negative Kahn tests are frequently found upon spinal fluids of incompletely treated cases of neurosyphilis. This is in direct contrast to that found in the results of the reaction upon blood serum, for in this case it has been repeatedly pointed out that the Kahn reaction appeared to be slightly more sensitive than the Wassermann.

LITERATURE

Kahn[2] reported the comparative findings of the Wassermann and Kahn tests upon 750 spinal fluids as follows: perfect agreement, 670 (394 positive, 276 negative) results stronger in Wassermann, 44, results stronger in Kahn, 26, and in 10 anticomplementary Wassermanns, satisfactory results were obtained with the Kahn.

Again Kahn[3] reported the findings of his test and the Harrison Wassermann method as follows: Four-plus reactions in the Wassermann 177, Kahn 223; 63 two-plus Wassermanns and 18 one-plus Kahns. Neither reaction gave any false positive results. These findings would indicate that the Kahn test was superior to this Wassermann technic.

*From the State Psychopathic Institute.
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Lederer[4] compared the results of the two tests upon 57 spinal fluids and found that the tests agreed upon 33 negative and 19 positives. There was one relative agreement and in four treated cases the Wassermann was more or less positive while the Kahn was negative. Using a modified Kahn technic he compared the two reactions upon 73 spinal fluids with the following results: 39 negative with both tests and 17 positives in perfect agreement, one fluid giving a weakly positive Wassermann gave a negative Kahn, and in 7 fluids the Wassermann was negative and the Kahn more or less positive.

Rabinovitch and Roberts[5] reported a series of 112 spinal fluids in which there was perfect agreement in 101 (63 negatives and 38 positives), and divergent results in 11 (4 stronger in the Wassermann, 6 in the Kahn), and one positive Kahn upon a fluid which gave an anticomplementary Wassermann reaction. They also observed that there was not any relation between the strength of the Kahn test and the amount of globulin present in the fluid.

Davenport[6] examined 487 spinal fluids with the Kahn tests and obtained negative reactions upon 5 cases of neurosyphilis in a group of 118 diagnosed cases. He also obtained negative reactions upon 23 incompletely treated cases. The results of the Kahn test upon the remaining fluids in his group were in agreement with the clinical findings.

Hull, Fry and Garwood[7] in 180 syphilitic cases found 135 positive and 45 negative Kahn reactions upon the spinal fluid. All but 14 of the negative tests were from patients who had received antisyphilitic treatment. On 229 fluids from patients classified as nonsyphilitic, 7 positive Kahn tests were procured. Of these 7 positive tests, 2 four-plus and 1 three-plus reactions were confirmed by positive gold sol reactions. The remaining had weak gold curves and weaker Kahn reactions. These authors conclude that the Kahn tests upon spinal fluid have a high degree of specificity in eliminating the possibility of cerebrospinal syphilis.

Germat and Serafimov[8] compared the Kahn and Wassermann reactions upon the fluids of 50 cases with clinical syphilis. They found complete agreement between the two tests in 94.5 per cent and divergence in 5.5 per cent. In the latter the Kahn gave more positive reactions. In progressive paralysis treated with malaria and sulphur in oil, the Kahn retained a stability of the positive reaction. Only one case with prominent improvement of the patient gave a negative Kahn test. They conclude that the Kahn reactions have a fairly simple technic and deserve great attention.

This brief review of the literature does not pretend to cover the subject, but is offered to demonstrate the diversified opinion as to the value of the two reactions.

TECHNIC

Because of the great dilution of the spinal fluid, it is first necessary to concentrate the reacting substances in the fluid by the precipitation of the globulin with ammonium sulphate and then testing the redissolved globulin by the Kahn reaction.[1] Since ammonium sulphate in sufficient concentration is capable of producing a precipitate with the antigen, more salt solution must

be used in the antigen dilution. This is usually between 0.3 to 0.4 c.c. more per 1 c.c. of antigen than the standard titer.

It is evident that this weakening of the antigen would more or less affect the strength of the reaction obtained with its use. Kurtz and Larkum[9] following the suggestion of Kahn and McDermott[10] have devised a technic which sufficiently removes the excess ammonium sulphate so that the regular serum titer can be used. After precipitating the globulin with the sulphate and centrifuging the specimen to throw down the precipitate, they pour off the supernatant fluid and invert the tube in saline, dipping it up and down, taking care that the saline does not touch the precipitate at the bottom of the tube. The air is allowed to escape by means of a glass tube reaching from the entrapped air in the tube through the saline into the open air of the room. This procedure is carried out in triplicate on each specimen and the globulin dissolved in 0.3, 0.2, and 0.1 c.c. saline. They found stronger reactions in those tubes containing the more concentrated globulin solutions.

We have been able to accomplish the removal of the excess ammonium sulphate by a technic which we feel to be simpler and to have less opportunity for mechanical accidents. In using the technic of Kurtz and Larkum unless great care is used in rinsing the tube in the saline solution, there is a possibility of losing some of the globulin because of splashing. Globulin is very soluble in saline, so, of course, any saline coming in contact with it would result in a loss of globulin and reacting substances.

MODIFICATION OF TECHNIC

Our method differs from the technic just outlined in that after the globulin has been thrown down by centrifuging, the supernatant fluid is carefully pipetted off, the tube is placed in an inverted position upon some filter paper and allowed to drain for one-half hour. Then the inner sides of the tube are carefully dried by means of a cotton applicator, taking great care not to disturb the precipitated globulin. The globulin is then ready to be redissolved and the regular Kahn technic continued, except that the antigen serum titer can be used. In our study we did not concentrate the globulin dilution, but followed the routine Kahn procedure for spinal fluid.

In removing the supernatant fluid it was found that if great care was used, it could be poured instead of pipetted off without affecting the end-result of the test.

EXPERIMENTAL STUDY

We have compared the results obtained upon 202 spinal fluids with the Wassermann (Craig technic[11]), standard Kahn, and modified Kahn (employing the serum antigen titer) reactions. This group consisted of 64 cases of untreated paresis, 90 cases of treated paresis, and 48 cases that were not considered to be paretics. The therapy used in the treated cases consisted of various fever therapies (diathermy, electric blanket, malaria and sulphur in oil) and tryparsamide. The spinal fluids were examined at a period ranging from two weeks following treatment to two years.

Considering the group as a whole there was a perfect agreement between

the three tests in 119 cases or 58.91 per cent (72 positives and 47 negatives). It would be very difficult to determine the relative agreement between the three tests, as many fluids had a relative agreement with two of the reactions but an absolute disagreement with the third test.

Chart 1 plots the curve of the perfect agreement of the spinal fluid Was-

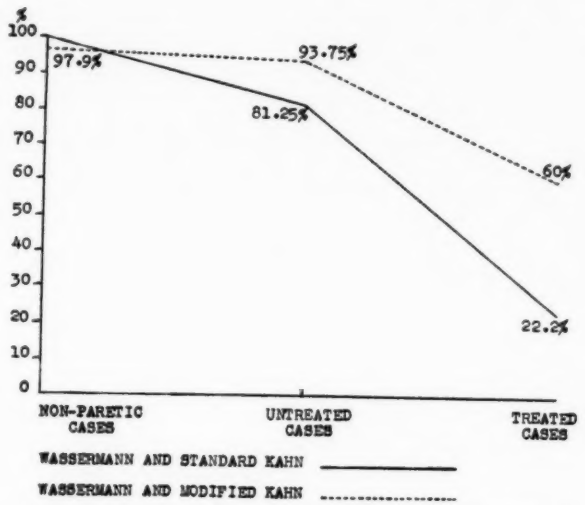

Chart 1.—Perfect agreement between spinal fluid Wassermann and Kahn reactions.

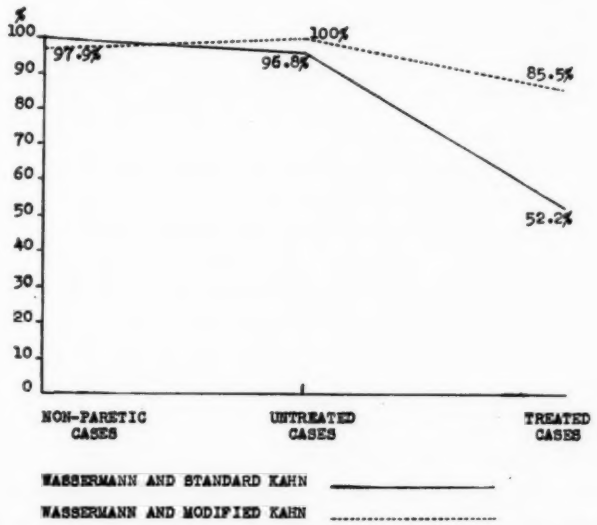

Chart 2.—Perfect or partial agreement between spinal fluid Wassermann and Kahn reactions.

sermann and the two Kahn reactions. There was 100 per cent agreement between the Wassermann and the standard Kahn in the 48 cases not diagnosed as paretics and having negative blood findings. In the 64 untreated cases of paresis, the agreement drops to 81.25 per cent or in 52 cases. In the treated cases this drops still further, or in only 20 of the 90 cases, or 22.2 per cent.

There was one false two-plus reaction by the modified Kahn test among

the 48 nonparetics, thus making a 97.91 per cent agreement between this and the Wassermann reaction. In the untreated cases there were 60 cases in which there was a perfect agreement between the two tests or 93.75 per cent. This percentage dropped to 60 per cent or a perfect agreement in only 54 of the treated cases.

Chart 2 considers the sum of the perfect and relative agreement between the tests. The Wassermann and the Standard Kahn reactions had 100 per cent agreement in the nonparetics; in the untreated cases, 62 cases or 96.87 per cent had either a perfect or partial agreement, and in the treated cases this dropped to 52.2 per cent or 47 cases.

The Wassermann and modified Kahn reaction had a perfect or relative agreement in 47 of the nonparetic or 97.91 per cent, in the untreated cases

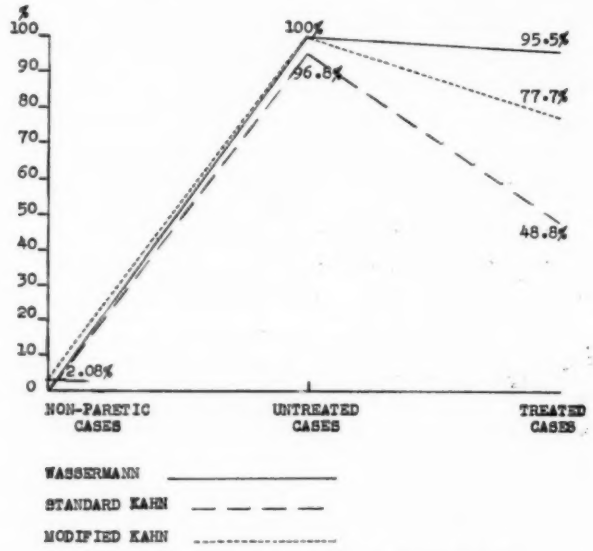

Chart 3.—Positive reactions in spinal fluid.

there was 100 per cent perfect or relative agreement and in the treated cases this agreement held true in 77 cases or 85.5 per cent.

Chart 3 plots the positive reactions of the three tests. There were no positive Wassermanns in the fluids of the nonparetics, 100 per cent of the untreated cases of paresis had positive spinal fluid Wassermanns and 86 cases or 95.5 per cent of the treated cases also had positive reactions with this test.

The standard Kahn reaction gave no positive reactions in the nonparetics; in the untreated cases, 62 or 96.87 per cent and in the treated cases, 44 or 48.8 per cent had positive reactions.

The modified Kahn test gave one false positive reaction in the nonparetic cases or 2.08 per cent, 100 per cent positive reactions in the untreated cases and in the treated cases 70 or 77.7 per cent had positive reactions.

Chart 4 demonstrates the comparative strength of the three reactions. The Wassermann and the standard Kahn both had all negative reactions in the nonparetics, but in the untreated cases the Wassermann was stronger than

the standard Kahn in 8 cases or 12.5 per cent. The standard Kahn was not stronger than the Wassermann in any of the untreated cases. In the treated cases the Wassermann exceeded the strength of the standard Kahn in 70 cases or 77.7 per cent, while the Kahn was stronger than the Wassermann in one case or 1.1 per cent.

Comparing the Wassermann and the modified Kahn reactions, we find that the modified Kahn had one false positive reaction in the nonparetics so that 2.08 per cent of those cases had stronger precipitation reactions than complement fixation. In the untreated cases there was a perfect agreement between the two tests, while in the treated cases the Wassermann was the stronger in 28 cases or 31.1 per cent. The modified Kahn was stronger than the Wassermann in only 6 cases or 6.6 per cent of the same group.

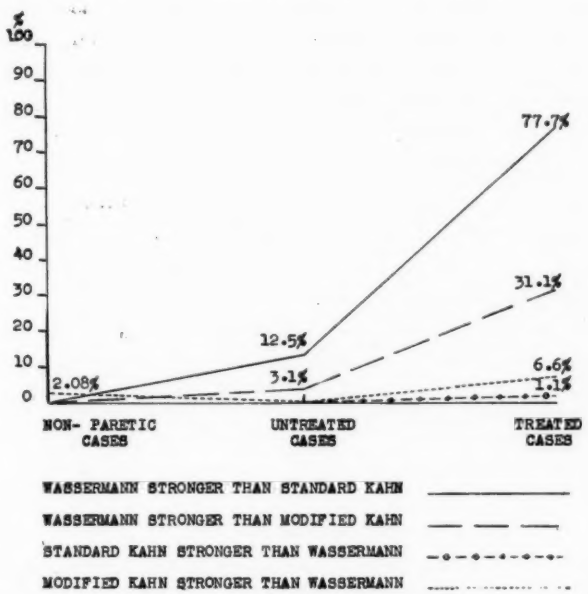

Chart 4.—Comparative strength of the spinal fluid Wassermann and Kahn reactions.

DISCUSSION

It will be noticed from the preceding data that our findings are in great disagreement with other authors. We feel this is due to the great number of treated cases included in our survey, because if only the nonparetic and untreated paretic cases are considered, there would be a relatively high percentage of agreement between the reactions. (Perfect agreement between Wassermann and standard Kahn, 89.2 per cent; Wassermann and modified Kahn, 95.5 per cent.)

Unfortunately the amount of fluid submitted for examinations was not great enough for us to follow completely the suggestion of Kurtz and Larkum and concentrate the globulin to a further extent than that of the standard Kahn technic. Undoubtedly, we would have found as they did, that more positive and stronger reactions could be obtained. However, in view of the fact that

we found one false positive among the 48 cases of nonparetics with the modified Kahn procedure, it would suggest that upon further concentration of the globulin, these false reactions would be found in even greater numbers. We feel that this method would be a dangerous procedure unless carefully controlled by a recognized Wassermann test and clinical evidence.

It is worthy of note that our findings indicate a false impression is prevalent if the Kahn reaction alone is used in determining the success of the treatment of neural syphilis: so many of these fluids had negative Kahns, although the Wassermann reactions remained positive. We definitely recommend the latter as the more delicate and reliable test upon spinal fluids.

CONCLUSIONS

1. The Wassermann reaction (Craig) upon spinal fluid gives a higher percentage of positive reactions in treated and untreated cases of paresis than the standard Kahn or the modified Kahn employing the serum antigen titer.

2. A new method is given to remove the excess ammonium sulphate from the precipitated globulin, which we feel is as efficient and more simple than the method of Kurtz and Larkum.

3. The agreement between the three reactions is relatively high in nonparetics and untreated paretics, but falls off rather sharply in treated cases.

4. The modified Kahn reaction employing the serum antigen titer gave a higher percentage of positive reactions in treated and untreated cases of paresis than the standard Kahn test.

5. One false positive reaction was observed among 48 nonparetics with the modified Kahn reaction, indicating a possibility of error if this test is used exclusively.

We wish to take this opportunity to thank Dr. Sidney D. Wilgus, Director of the State Psychopathic Institute, for his interest, helpful guidance and suggestions in the preparation of this study.

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SPECIAL RACK FOR SMITH FERMENTATION TUBES AND "U" TUBES*

PAULINE EPSTEIN, NEW YORK, N. Y.

ONE of the means of identification and differentiation of various strains of bacteria is the sugar reaction test, for which a Smith fermentation tube is used. The latter is filled with sugar broth into which the organism to be tested is inoculated. The amount of gas produced by the organism as measured by its volume in the arm of the tube, as well as the production of acid, as

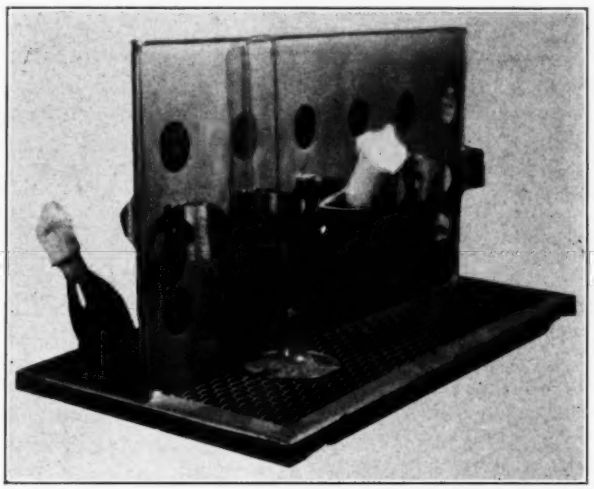

Fig. 1.

evidenced by the change in the color of the sugar broth, frequently facilitates the classification of different microorganisms. However, due to its specific and cumbersome form, the wide use of the Smith tube has been found difficult.

In the process of the fermentation test the tube is subjected to numerous manipulations:

A. Sterilization of the tube itself in hot air sterilizer.

B. Filling it with culture media and sterilization of the media in the autoclave or Arnold.

C. Inoculation with the organism to be tested and incubation for its growth, etc.

In the course of this handling the tube, because of its thin stem, too often breaks. In addition, due to its instability, the tube frequently tilts or falls and displacing part of the gas in the arm by the broth, disturbs the test. To avoid the breakage and to secure the results of the test, I devised a rack which

*From the Department of Bacteriology, New York University and Bellevue Hospital Medical College.

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has been successfully used for some time in our laboratories. This rack consists of a base and a vertical plate. The base, 13.5 inches by 7.5 inches, is made of a metal frame and a wire mesh, which has 16 openings per square inch, thus allowing free circulation of steam or heated air during sterilization.

The vertical plate, 7.5 inches by 13.5 inches is centered at the base. Attached to the plate, centrally located, are six clasps at 2¼ inches spacing on each side.

Directly above and below each clasp there is a ¾ inch diameter opening punched through the plate. The sides of the clasps also have ¼ inch diameter

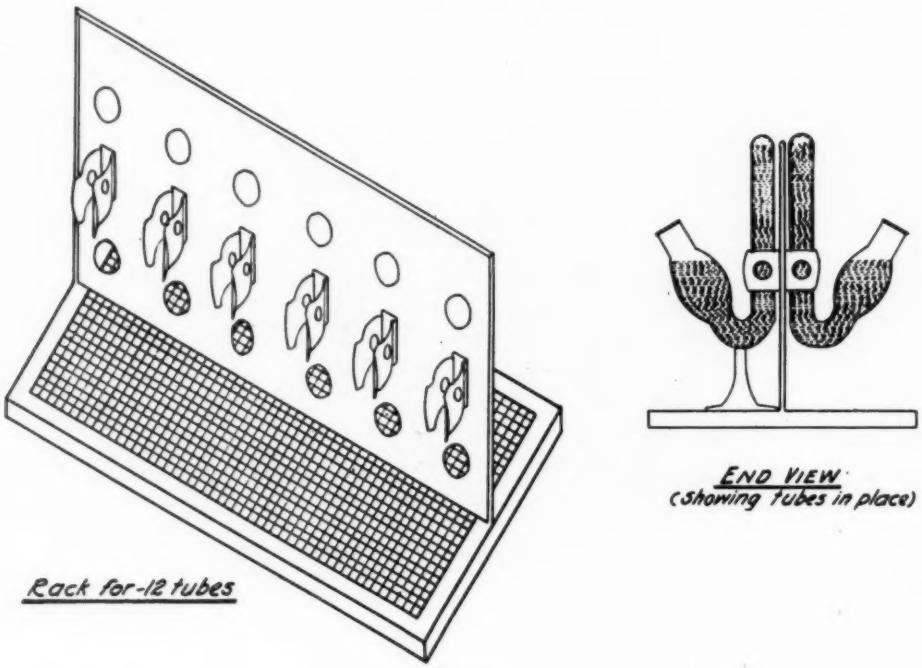

Fig. 2.

openings. The holes in the plate as well as those in the clasps enhance further circulation of air and the maintenance of a uniform temperature through the entire surface of the rack.

The rack is designed to hold "U" tubes as well as Smith fermentation tubes. In either case, the long arm of the tube is placed in the clasp, while in the Smith fermentation tube the stand rests on the base of the rack, thus affording a double support, as shown on the end view of the rack.

Monel metal was used in the construction of the rack because it is rustproof and preserves its appearance. It is a solid unplated metal, white, very hard and can therefore be subjected to various temperatures.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

DERMATITIS, Contact, Patch Test in the Diagnosis of, Ayres, S. Jr., and Anderson, N. P. Ann. Med. 6: 1161, 1933.

The advantages of the patch test in determining the etiology of certain cases of dermatitis are mentioned, together with a description of the method of applying the test.

Twenty-four cases of eczematoid dermatitis are reported, some of them in detail, in which the etiology was established by means of the patch test.

The method advocated by the authors is as follows:

If the substance is a chemical it should be applied in approximately the same dilution to which the patient has been previously exposed. Leaves of plants and shrubs should be as fresh as possible. Dry powders may be moistened slightly with water. The substance to be tested is placed on a small square of cotton about the size of a postage stamp which is then applied to the flexor surface of the forearm, or other suitable area, and covered with a square of some impervious material such as cellophane, which should be about four times as large as the test area. This external covering is then held firmly in place by wide strips of adhesive tape. The space on all sides between the edges of the central cotton square and the edges of the cellophane is sufficient to prevent the adhesive tape from coming too close to the test area. Otherwise the mild temporary irritation which usually results from the adhesive tape might interfere with an accurate interpretation of the reaction. The patch test is allowed to remain undisturbed for twenty-four hours unless discomfort from irritation justifies its removal before this time. A positive reaction is characterized by a sharply defined square of redness corresponding in size and shape to the central test area. Papules and vesicles are also usually present, corresponding to the appearance of the original eruption. Evidences of the reaction can usually be noted for three or four days or longer after the test is applied. In rare instances the reaction may not develop for forty-eight hours after the test is applied.

The test is simple, inexpensive, and closely simulates the natural conditions under which the dermatitis developed. It is usually very accurate. A few cases have been reported in which only certain areas of the skin were hypersensitive so that in the case of a dermatitis of the face, a patch test on the arm was negative while one performed on the face after the eruption had subsided was strongly positive. Such instances are probably uncommon, but the possibility should be borne in mind.

BLOOD PROTEINS, A Micromethod for the Quantitative Estimation of, Medes, G. Am. J. Clin. Path. 3: 439, 1933.

One and five-tenths cubic centimeters of blood from the ear of a rabbit is allowed to drip directly into a centrifuge tube containing powdered potassium oxalate, with which it is thoroughly mixed. The tube is centrifuged at 3,500 revolutions a minute for four minutes. The plasma is syphoned off through a fine glass syphon into a small straight-sided vial containing a 2-holed rubber stopper through one hole of which passes the syphon, and through the other, a short glass tube connected by rubber tubing to a glass mouthpiece. By this arrangement the stream of plasma through the syphon can be regulated and the plasma removed from the mass of cells to almost the last drop. The plasma is then distributed to previously prepared 10 c.c. volumetric flasks by means of a Folin tube for micro determination of blood sugar, accurately graduated in 0.1 and 0.2 c.c. graduations.

Preparation of Flasks: The following amounts of anhydrous Na_2SO_4 are weighed on an analytical balance into a series of 10 c.c. flasks: (1) none, (2) 1.07 gm., (3) 1.42 gm., (4) 1.78 gm., (5) 2.204 gm. Into Nos. 2, 3, 4, and 5 are added 0.4 c.c. of a buffer solution to be described below. To all five tubes is added water at 35° to just below the 10 c.c. mark.

The flasks are stored in an incubator at 35° in order to maintain the Na_2SO_4 above its eutectic point. When ready for use, 0.1 c.c. plasma is measured into each of Nos. 1, 2 and 3, and 0.2 c.c., into Nos. 4 and 5, the volumes made up to 10 c.c. with warm water and the flasks returned to the incubator for three hours.

Composition of Buffer (Berglund): 29.6 c.c. of 0.2 M carbonate free NaOH, 50 c.c. 0.2 M of KH_2PO_4, distilled water (carbonate free) to make 100 c.c. This should give a solution with P_H 7.0

At the close of the three-hour period, the contents of Flasks 2, 3, 4, and 5 are filtered. A fine smooth filter paper 6 cm. in diameter is employed. The filtrates are collected in graduated centrifuge tubes; 9 c.c. can usually be procured. At the same time 9 c.c. of the contents of Flask 1 are transferred to centrifuge Tube 1. To each of the five tubes is added 1 c.c. of 50 per cent trichloracetic acid and the contents thoroughly mixed by means of a fine glass rod which is then washed off with 4 per cent trichloracetic acid. The centrifuge tubes are allowed to stand for ten minutes at room temperature, and are immersed for ten minutes in a water-bath at 50° C. They are centrifuged at 3,500 revolutions per minute for twenty minutes, after which the supernatant fluid is decanted and the precipitate dissolved in one or two drops of 10 per cent NaOH stirring thoroughly with a fine glass rod and spreading the NaOH over the entire inner surface of the centrifuge tube. The contents are then made up accurately to the original volume (9 c.c.).

The nitrogen of each of these solutions is determined by Folin's micro Kjeldahl method, employing 2 c.c. of each. Occasionally 3 c.c. may be required for some of the lower fractions of globulin.

Computation:

$$S \times \frac{20}{R} \times \frac{10}{X} \times \frac{100}{Y} = N$$

where S = value of standard, R = reading of colorimeter, X = 0.1 or 0.2 c.c., according to the fraction being ashed, Y = No. of c.c. ashed, and N = milligrams protein nitrogen in 100 c.c. of plasma. To obtain the grams of protein per 100 c.c. of plasma, multiply by 6.25 and divide by 1,000. Total protein equals protein in Tube 1; fibrinogen equals Tube 1 minus Tube 2; euglobulin equals Tube 2 minus Tube 3; pseudoglobulin I equals Tube 3 minus Tube 4; pseudoglobulin II equals Tube 4 minus Tube 5; albumin equals Tube 5. In case values of only total protein, fibrinogen, globulin and albumin are desired Tubes 3 and 4 are omitted.

MALARIA, A Method of Making Slide Smears From Female Anopheles, etc., Barraud, P. J. Ind. J. Med. Res. **21**: 457, 1933.

The advantages of the technic described may be summarized as follows:

1. The method combines, in the large majority of preparations, the advantages of dissection of the salivary glands, with those of Sergents' method for detecting the presence of sporozoites in the body-fluid of mosquitoes.

2. The insect host is preserved for identification and for reference in the future.

3. The method can be carried out rapidly in the field where laboratory facilities, and sufficient time, for carrying out dissections in the ordinary way, are not available.

4. The preparations can be made by an assistant not necessarily trained in the detection of sporozoites, or in the identification of mosquitoes.

5. A microscope is not necessary for making the preparations.

The apparatus required is as follows: A supply of test tubes with wool plugs; a supply of microscopical slides; two needles, or pins (preferably mounted in handles); some staged tubes; a supply of small double-pointed entomologic pins and a pair of entomologic forceps. A chloroform tube, or a supply of chloroform, is also useful. The slides may be prepared previously by marking off three spaces by drawing transverse lines with a diamond, about one inch apart, and numbering these consecutively.

The mosquitoes are caught in test tubes. When a preparation is to be made, kill the

insect with chloroform, or by blowing tobacco smoke into the tube. When dead, turn the specimen out on to a slide. Arrange the whole mosquito on a numbered square, on its left side, with the head pointing away from you. Do not add any saline, or other fluid, as this is not required. Do not remove the wings or legs, and endeavor to carry out the following manipulations with as little damage as possible to the scaling, etc. Take a needle in the left hand and place the shaft of this flat across the front of the thorax. Press down with this so as to cause the thin chitin of the front of the thorax below the neck (''prosternum'') to bulge out considerably.

The salivary glands lie just within this thin sheet of chitin. With a needle held in the right hand cut into this part. Draw out, and cut off, the tissues which will protrude, and by continued pressure with the left hand needle at the same time, squeeze out some of the body fluid on to the slide. Endeavor to avoid severing the neck, or detaching the front legs. Tip the mosquito on to a piece of paper, and with a needle rapidly mash up and make a small circular smear, on the numbered square, of the tissues and fluid left on the slide. Dry this by waving the slide in the air. It should be noted that the dissection and smear are made without any admixture of saline. When opportunity occurs the smear should be fixed with methyl alcohol or absolute alcohol, and the slides stored for staining and examination, which may be done at any convenient time later.

Pin the mosquito and mount it in a staged tube, marking on the stage the number corresponding to the smear on the slide. Observations on the locality, the nature of the place where the specimen was collected, the date, and any other details considered worth recording, should be entered in a notebook at the time.

Before making another preparation clean the needles thoroughly.

GONOCOCCI, Cultivation of, Philadelphy, A. Wien. Klin. Wchnschr. **46**: 1052, 1933.

Two parts sterile human or horse blood are mixed with one part of distilled water in a flask. The flask is closed with a sterile glass stopper and shaken vigorously for three minutes to prevent coagulation. It is then kept in the incubator at 60° for half an hour. After this time a foamy mass of fibrin collects at the top, while the dark red blood serum is fluid. This is mixed in a proportion of 1 to 2 with nutritive agar at 60° with a 2 per cent agar content and a P_H of 7.3 to 7.5. The nutritive medium is ruby red and transparent.

The author made 415 cultures from 108 patients suspected of gonorrhea. In 354 instances the cultures agreed with the microscopic findings. In 29 instances the cultures showed growth of gonococci though no gram negative gonococci were found microscopically. In four cases that were doubtful microscopically cultures gave positive results. In three cases that were positive microscopically there was no growth of the cultures. The results of cultivation on blood serum agar were at least as good as those on ascites agar.

IODINE, An Improved Micromethod for Estimating, Patnaik, M. Ind. J. Med. Res. **21**: 237, 1933.

The sample for analysis is rendered alkaline. Fats and oils should first be saponified with 10 per cent alcoholic potash, then evaporated to dryness in a porcelain basin and dissolved in hot water. Cereals and vegetables should be heated with alkali till a complete paste is obtained. Whatever the sample may be it should be converted into an easily workable form. To such an alkaline solution 7 per cent solution of $KMnO_4$ is gradually added and while stirring with a glass rod the porcelain basin is heated over an asbestos protected flame. When the oxidation is complete and there is a slight excess of $KMnO_4$, absolute alcohol is added slowly until the excess of $KMnO_4$ is reduced. It is then cooled and filtered quantitatively and the hydrated manganese dioxide removed. The filtrate is then evaporated to dryness with a small amount of K_2CO_3. The residue which always contains some nonoxidizable disintegration product, besides potassium iodate, is heated over a protected flame till charring takes place without the evolution of smoke (some amount of smoke is evolved from fats and oils at this stage—the amount of smoke being very much less than that in the usual incineration method). It is then heated slowly over a mild flame till the organic matter is

disorganized. During this process of heating all the iodate coming in contact with the finely divided carbon is reduced to iodide. It is then made into a paste and extracted and estimated as usual.

If during incineration the charred mass remains in a molten state due to an excess of potash, a requisite amount of K_2CO_3 should be added to render the final product suitable for alcoholic extraction.

For the determination of iodine from pure $KMnO_4$, 50 gm. of $KMnO_4$ is taken, and after reduction with alcohol it is filtered quantitatively. The filtrate is evaporated in five nickel dishes after addition of 3 gm. of K_2CO_3 to each dish. Then before the evaporation is complete one gram of sugar (there being no iodine in sugar) is distributed among the five dishes. The rest of the process remains the same. In every determination the amount of iodine present in the $KMnO_4$ used up is subtracted in order to get the actual value.

SYPHILIS, Flocculation Tests For, Mugrage, E. Am. J. Clin. Path. 3: 421, 1933.

The following tests were compared in 1,000 blood specimens: Flocculation tests: Eagle, Hinton, Kahn, Kline, Meinicke (M.K.R.), and Miller (M.B.R.). Complement fixation tests: Kolmer quantitative, and a double antigen test.

This series has shown the advantage of performing a complement fixation test and a flocculation test on every blood specimen as supplemental to each other.

The flocculation tests have a definitely higher degree of sensitivity than the complement fixation tests in treated and latent cases of syphilis.

The majority of the tests concerned have a high degree of specificity.

All eight tests require a high degree of technical skill, and increasing experience with any test enhances its value.

SERUM PROTEINS, Diagnostic Value of Changes in Kala Azar, Mudaliyar, M. R. G., Sundarum, S. K., and Ramachandran, A. S. Ind. J. Med. Res. 21: 361, 1933.

The serum protein changes in kala-azar while not absolutely diagnostic of the disease offer distinct help in diagnosis of kala-azar in doubtful conditions.

The essential changes in serum proteins in kala-azar are a fall in albumin with considerable rise in total globulin, associated with increase in the relative proportion of euglobulin. A similar, but less striking change, has previously been recorded by Lloyd in secondary syphilis.

Serum protein changes in a number of miscellaneous conditions have been recorded.

B. PERTUSSIS, Rapid Agglutination Technic Applied to, Kendrick, P. L. Am. J. Pub. Health 23: 1310, 1933.

Antigen.—The forty-eight-hour growth of *B. pertussis* on a Bordet-Gengou slant is transplanted to a half Petri plate of Bordet-Gengou medium. After forty-eight hours' incubation, the growth is removed with a stiff, bent needle and emulsified in 1.5 c.c. of physiologic salt solution. The suspension is filtered and adjusted, if necessary, to a turbidity of approximately 10 billion organisms per c.c. by comparison with a standard.

Filtration is an important step in obtaining satisfactory smooth suspensions. Test tube filters are prepared for this purpose. A thin layer of moistened cotton is shaped around the finger, placed on a simple copper wire netting support, and inserted in a 5 × ⅝ tube, the free end of the wire strip being bent to hook over the lip of the tube. The cotton is molded against the walls of the tube by means of a wooden applicator. These filter tubes are plugged with cotton and sterilized. The filter is easily removed after use and the plug reinserted.

For a larger filter, a layer of gauze and cotton is wrapped around the outside of a square of wire netting cut from a bias strip and shaped to fit inside the nick of the bottle or flask of the desired size. The filter is suspended by two wires which cross under it, pass through the netting, and are hooked over the lip of the flask.

Antiserum.—For preparation of a diagnostic serum, a rabbit is injected intravenously with a 10 billion per c.c. suspension prepared from a forty-eight-hour growth of a recently isolated *B. pertussis* culture to which merthiolate 1:10,000 has been added at least twenty-four hours previously. The injection doses are 0.4 c.c., 0.8 c.c., and 1.0 c.c., respectively, at three- to four-day intervals. About 1 week after the last injection, the agglutination titer (equivalent, as explained below) has been around 1:20,000 in the six different rabbits treated by this method. The serum dilutions for agglutination tests are chosen according to the particular conditions. For testing cultures isolated by the cough plate method, the dilutions usually employed are 1:10, 1:100, 1:500, 1:750, 1:1,000, 1:1,500, and 1:2,000. For uniformity, the same dilution scheme is always employed and separate pipettes are used for the different dilutions.

The agglutination test—one-tenth c.c. of each serum dilution is mixed with 0.1 c.c. of antigen, the measurements being made with 1 c.c. pipettes graduated in tenths. For an antigen control, 0.1 c.c. of saline is mixed with 0.1 c.c. of antigen. The mixtures are shaken by hand for three minutes. For the shaking process, the racks are rocked at the rate of approximately 60 back-and-forth motions per minute and in such a way that the contents flow up the walls of the tubes for about three-quarters of their length. After the shaking period, physiologic salt solution is added for greater ease in reading. Because of its convenience, the Hipple pipetting apparatus, used in the Kahn test and set to deliver 0.5 c.c. of saline, is ordinarily employed.

Reading the tests—the tests are read immediately after the addition of saline and each tube recorded as −, ±, +, ++, +++, or ++++, according to the degree of agglutination. In the interpretation of the results in comparison with those given by other workers, a question arises because the final dilutions in the rapid test are not strictly comparable with those ordinarily given. This is of small consequence, however, provided the method of expressing the results is clearly stated. In the 0.2 c.c. mixtures of the rapid test, the series of serum dilutions 1:10 to 1:2,000 would give a series of final dilutions from 1:20 to 1:4,000. Any particular final dilution in the usual 1 c.c. test would contain five times the actual quantity of serum and antigen contained in the same dilution of the 0.2 c.c. rapid test. Therefore, if based on the actual quantity of serum present in the mixture, the series of final dilutions 1:20 to 1:4,000 of the rapid test would be equivalent to a series 1:100 to 1:20,000 in the usual 1 c.c. test.

TYPHOID BACILLI, Isolation of From Water and Sewage, Wilson, W. J. Brit. M. J. 3794 (Sept. 23), 560, 1933.

The method following is described as very efficacious:

To 100 c.c. of 3 per cent nutrient agar were added 10 c.c. of a stock glucose-phosphate-sulphite-bismuth mixture, and 1 c.c. of a 1 per cent solution of brilliant green. Equal volumes (25 c.c.) of the medium, cooled to 55° C., and water were mixed and poured into Petri dishes. After the medium had set and been incubated, black colonies consisting of typhoid bacilli developed in its substance. These were subcultured on to MacConkey plates, and cultural and serologic tests applied.

The bismuth media are very easily prepared when a stock solution is available, and such stock solutions keep for months. Dissolve 6 grams bismuth-ammonio-citrate scales in 50 c.c. of boiling distilled water, and neutralize by the addition of about 2 c.c. of 10 per cent caustic soda. Mix with a solution obtained by boiling 20 gm. sodium sulphite anhydrous in 100 c.c. of water, and then, while the mixture is boiling, add 10 gm. of sodium phosphate anhydrous. To the sulphite-bismuth-phosphate mixture, when cool, add a solution of glucose obtained by dissolving 10 gm. of commercial glucose in 50 c.c. of boiling distilled water. For preparation of old standard medium, add to 100 c.c. of a hot melted 3 per cent nutrient agar 20 c.c. of stock mixture, then 1 c.c. of an 8 per cent solution of ferrous sulphate crystals in water, and 0.5 c.c. of a 1 per cent solution of brilliant green in distilled water; pour into Petri dishes, and when the medium has set inoculate the surface. Isolated colonies of *B. typhosus* and *B. para-typhosus* are black, the typhoid colonies usually appearing within twenty-four hours, and the paratyphoid within forty-eight hours.

UNCINARIASIS, Acute From Massive Infestation and Its Implications, Ashford, B. K., Payne, G. C., and Payne, F. K. J. A. M. A. 101: 843, 1933.

In the discussion of an outbreak following prolonged bathing in polluted water the authors comment that the secret of what happened to these patients is found in a study of the leucocytes.

First, infestation, when sufficiently massive, produces leucocytosis, and this leucocytosis is eosinophilic. In every one of these cases this occurred and on these abnormalities, with a history of ground-itch, sudden loss of strength and color, and, generally, diarrhea, the correct diagnosis should be strongly suspected, if not actually made clinically, as in this instance it was made. In these cases, in from one to two months after infestation, a high leucocytosis was found already developed. It oscillated for two or three months and then, throughout nine months, gradually sank to near or quite normal.

But if the leucocyte count is striking, the eosinophile percentage is still more so. When the patients were first examined, this percentage was found to lie all the way between 14 and 69. When most of the worms in the intestine had been expelled, the percentage arose; but this was not on account of the expulsion of the worms. There is, we believe, a more reasonable explanation: There is a growing feeling that eosinophilia is a phenomenon related to the disintegration and absorption of foreign protein. The old idea that eosinophilia in uncinariasis is due to a toxin produced by the worm can no longer be accepted in its literal sense. The eosinophilia rises when the larval mortality in the tissues rises.

BIOPSY, Present Status of, McGraw, A. B., and Hartman, F. W. J. A. M. A. 101: 1205, 1933.

The worth of the biopsy in diagnosis, prognosis and as an index for treatment is at present thoroughly and soundly established.

Properly conducted, its lack of danger is likewise established. Its few contraindications are well known and recorded.

Its status as a technical procedure, though clearly allowing of future improvement, has attained a satisfactory level of combined simplicity in procedure and materials and of reliability in preparation.

Its opportunities for future development and practice under optimum conditions lie largely in stimulation of interest and furnishing of facilities for more pathologists and surgeons to devote the time and hard work necessary to perfect themselves in the difficult field of tumor pathology.

POLYCYTHEMIA, Benign Familial, Spodaro, A., and Forkner, C. E. Arch Int. Med. 52: 593, 1933.

Observations on the blood, together with other clinical studies, of ten members (two generations) of a family in which polycythemia and splenomegaly were present are reported. The existence of polycythemia in an earlier generation of the family is suggested by the history.

This family differed from those previously described in that characteristics of polycythemia vera, other than the increased number of red blood corpuscles and enlargement of the spleen, were not present.

The term "benign familial polycythemia" is suggested to distinguish this condition from polycythemia vera and from polycythemia due to recognized causes.

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EDITORIAL

Some Pitfalls in the Study of Sex Hormones

ONE of the most diligently cultivated fields of scientific investigation at the present time is that of the interrelations between the gonads and the anterior hypophysis, the fruits of which have already amassed a large volume. As is always the case, much of this literature is confusing in its conclusions, with, oftentimes, a blind following of methods already outmoded. In the following paragraphs an attempt will be made to evaluate some of this literature in terms of actual accomplishments, with a view of aiding further work along this line. The whole subject has been amply reviewed by many able investigators and a duplication of this will not be attempted, the aim being rather to correlate certain phases of the work.

As one examines the literature it is evident that the same criteria of the efficacy of administration of the sex hormones is fairly general, the weights of the gonads of immature animals which have received injections for a short period, usually two or three days, with the opening or closure of the vagina. In very

many cases, particularly with routine assaying of extracts, the latter test only is used, many investigators using the same animals repeatedly, with short intervals between each test.

It has been shown (Lipschütz, 1932; Swezy, 1933a; Evans et al., 1933; Fluhmann, 1933) that the ovary has no necessary relation to the vagina when hormones are administered experimentally, cornified cells occurring with the ovary in all stages of change between normal and excessive luteinization, and with or without large follicles, hence this test gives evidences only of the presence of the single factor responsible for vaginal changes. Lipschütz further showed that these vaginal changes may take place with no corresponding changes in either uterus or ovary. Much evidence is also accumulating to show that this factor is not identical with folliculin, progestin, the growth, follicular growth, or luteinizing hormones, and is widely distributed, occurring even in plants. As was first demonstrated by Stockard and Papanicalaou (1917), the different stages of the normal estrous cycle of many of the lower mammals are characterized by clear-cut changes in the cellular contents of the vagina. Much experimental work has also shown that these changes are directly due to the action of hormones. The work shows equally well, however, that decreasing or increasing these hormones completely upsets the normal relations between them, resulting in conditions which diverge widely from the normal, not only in the ovary, but in the uterus, vagina, and even in the hypophysis itself. Under such conditions it is too much to expect that the vagina will still reflect the changes in the ovary. That this is true is abundantly proved by the results of much published work, in which all conditions of the ovary are associated with estrous smears as well as with diestrous ones. This fact that it does not give reliable information concerning the state of the ovary and uterus makes the vaginal smear method an unsuitable one for sole reliance as an assay test for sex hormones. In his recent review of the biologic assay of the sex hormone of the ovary, Marrian (1933) omits all mention of any other test for this hormone. That it is the sole test in so much of the work with hormones may well account for much of the variable results reported.

The practice of using the same animals repeatedly after a few days' interval has an even greater fault. The recent work of Emery (1933) shows that the ovaries of rats receiving injections of two castrated rat hypophyses on two successive days, reach a maximum size at twelve days and remain larger than the controls for about two months, when rats twenty-five days old are used. Since this is the usual age and length of treatment for experimental rats, it is evident that they will not be in optimum condition for some time after the initial treatment, as it is doubtful if results exactly comparable to those obtained in animals with ovaries in the normal immature state will be obtained in animals with ovaries already extensively luteinized. Even when no vaginal changes are found in these animals, it has been shown that changes in both ovary and uterus may take place (Evans et al., 1933), which might render their use a few days later of questionable value. The results reported by Selye and Collip (1933) show that early luteinization (day sixteen) will prevent the usual reaction of the ovary at a later date (day twenty-six) with continued injections. The work of Evans indicates the same possibility. The use of such animals may account for

much of the variable results obtained from different samples of these hormones put out by commercial laboratories, but the practice is by no means confined to such laboratories, as it is usually recommended for all routine work. Coward and Burn (1927) used animals not more frequently than once a fortnight, while Allen et al. (1930) used rats once a week. Coward and Burn found that out of 50 rats used three times, only 19 gave the same result each time. These were ovariectomized. Since these hormones affect the ovary mainly, it is probable that the differences would have been greater in normal animals. It is doubtful whether the disadvantages of using animals repeatedly, without a sufficient time elapsing for full recovery, can be compensated for by using large numbers of animals. This might be of little importance with many experiments, but for careful analytical work it is the attention to such small details which assures success.

The use of the weight of the ovary as an assay test is also open to criticism, since this is no infallible guide to the kind of change induced by hormone treatment, though excessive weights are nearly always associated with extensive luteinization. In a recent article Van Dyke and Wallen-Lawrence (1933) attempt to determine the differentiation of the follicular growth and luteinizing hormones by the weights of the ovaries induced in immature rats, without reference to the possible presence or absence of lutein cells in them. It is necessary that some follicular growth take place before a noticeable change in the weight of the ovary is obtained. But, since the luteinizing hormone may induce the formation of lutein cells from the germinal epithelium and tunica albuginea (Swezy, 1933a), it seems certain that evidences of the presence of this hormone may be found with but little change in either size or weight of the ovary. Selye et al. (1933) induced luteinization in the thecal cells of the small follicles of the ovary of the rat, beginning about day thirteen, with no macroscopic change in the ovary, with pregnancy extracts, and in the guinea pig without accompanying follicular growth. Any attempt therefore to determine the presence or absence of the luteinizing hormone in any preparation would involve a careful examination of stained sections of the ovary, even though no response had been elicited by the treatments in the vagina or in the weight of the ovary. In fact this seems more necessary in the case of the latter animals, as a pure luteinizing hormone would thus be demonstrated.

A considerable amount of follicular growth may also be found without a significant increase in the weight of the ovary. The presence of follicular growth hormone in small amounts could be detected only by the same means as has been suggested for the luteinizing hormone. Between this stage and that of extensive luteinization or the production of large follicles in great numbers, however, it is not always possible to determine from macroscopic examination what structures are predominant.

The relation of estrin, or factor inducing cornification in the vagina of rodents, to folliculin, or factor inducing growth in the endometrium, is one fraught with much confusion. In the normal animal these two factors seem to occur at approximately the same time in most cases, though this does not always hold true in the cat (Evans and Swezy, 1931). Under experimental conditions the preponderance of evidence indicates that the factor inducing cornifi-

cation is not identical with that inducing growth in the uterus, though the idea that they are one and the same seems to be prevalent. This acceptance of their identity has resulted in the widespread use of the vaginal smear test as the important one in the assay of these hormones.

Many investigators believe that a single hormone may be responsible for the changes in uterus and vagina, the relation being a quantitative one, yet, *in the normal animal, maximal growth of the endometrium is associated with cornification, this never occurring during the initial stages of growth when a smaller amount of hormone is presumably present.* Further evidence against this assumption is found in the work of Evans et al. (1933), in which all stages of growth of the uterus from medium to an estrous type and ovaries with medium and large follicles are present in rats treated with hypophyseal hormones and the vagina still closed. On the other hand, Lipschütz obtained vaginal changes with the administration of hypophyseal extracts but no changes in either ovary or uterus. The presence of cornification with no medium or large follicles, has been noted by a number of investigators, as well as ovaries filled with large follicles and a diestrous smear in the vagina. With these results in both normal and experimental animals, there seems to be no grounds for assuming that a single factor is involved in the changes in the vagina and uterus. Two separate factors are indicated.

Allen et al. (1930) found that the amount of estrin present in human corpora was highest after recent ovulations, days thirteen to sixteen, and lowest in ovaries with older corpora, in two cases at days twenty and twenty-two, and that there was an abrupt drop with menstruation. It is well known that in the first half of the ovarian cycle following ovulation, there are no large follicles in the ovary, these reaching a large size only toward the end of the cycle. Since ovulation seems to occur in the middle third of the cycle in the majority of cases, these relations would mean that the eight to ten days preceding menstruation would find only small to medium-sized follicles in the ovary. Frank and Goldberger (1926) find, however, that estrin reaches its greatest concentration near the first day of menstruation, the early part of the cycle showing only a small amount, with an increase from day ten to fifteen. If these were not cases in which ovulation occurred near menstruation, these results would suggest that the greatest concentration of follicular secretions came at a time when there were apt to be only small to medium-sized follicles in the ovary. This assumption is contradictory of much work on the lower mammals, since here the greatest concentration, as shown by the only critical test, the growth of the uterus, comes immediately preceding or coincident with ovulation. At first sight this would seem to mean that these physiologic processes and hormones differ radically in the human from those of the lower mammals, yet a considerable body of work indicates that they are very similar. Frank and his coworkers used only the presence of cornification in the vagina as the test of these hormones.

Cornification has no recognized place in the human cycle, hence the factor for it, found in a wide range of preparations, including plants, would seem to have no invariable relation to the ovarian cycle, while the factor causing growth in the endometrium does have a constant and invariable relation. The regularity of the uterine cycle in the human being, with its dependence on folliculin, is as

constant as may be found in the rat or guinea pig. There are no evidences yet brought out indicating a different hormonal relation. A clarification of these relations and the hormones associated with them can be obtained only by more critical analyses of the experiments conducted with them. In the light of present data it would seem expedient to restrict the use of the term estrin to the factor related directly to the vaginal changes, and, what is more important, to restrict the vaginal smear test to the assay of estrin, and not use it as a valid test for other hormones.

It has been pointed out elsewhere (Swezy, 1933b) that in all experimental work on normal animals, the hypophysis of the test animal itself largely decides the results when sex hormones are being studied. Whether this is true of other hormones also must be determined by further work. This result seems to be due to the presence of an activator in some preparations, including human pregnancy extracts and the hypophysis of the infantile rat, which is able to stimulate the hypophysis of the host to increased hormone production. This conclusion is supported by a number of experiments from different laboratories. The difficulties of determining the hormone content of any preparation are thus increased, since this can be done accurately only when the animal's own hypophysis is eliminated through hypophysectomy. This does not disparage the use of normal animals for such experiments, however, as these are a vital necessity in gaging the therapeutic value of such preparations, but it does suggest the necessity of basic work under carefully controlled conditions, and such conditions are not obtainable in the normal animal, its own hypophysis being the disturbing factor.

In judging the value of these preparations the "gonad-stimulating potency" has been one of the main tests. In the rat this potency is measured by the weight of the ovaries produced, great potency producing heavy ovaries, filled mainly with luteal tissue. The luteinizing hormone, which brings about this result, checks ovogenesis and the growth of follicles (Swezy, 1933a) and produces ovaries having a tumor-like metabolism (Szarka et al., 1933). In the normal animal the corpora lutea, so far as present data indicate, have little or no function beyond inducing the secretion phase in the uterus and the maintenance of pregnancy. It is therefore a serious question of what value the administration of the luteinizing hormone, which has, as its chief function, the induction of the formation of this luteal tissue, may be in the nonpregnant individual. Its disadvantages would seem to outweigh any possible benefits. On the other hand, the hormone which stimulates the growth of the follicles, resulting in the secretion of folliculin with its reaction on the hypophysis itself, would seem to have a greater value as a therapeutic agent. This aspect of the effects of the "gonad-stimulating hormones" needs further study before the clinical importance of these preparations can be adequately tested, and some other criterion must be found for evaluating the extracts which will not stress the luteinizing hormone as the important one, a position which it holds in present methods of testing them for potency. In this connection the suggestion of Leonard (1933), that pregnancy extracts should be combined with the hormones of the hypophysis when "great ovarian stimulation" is desired, further emphasizes the need of caution on this point, as the desirability of ever producing such great ovarian

stimulation is open to very grave doubt. These heavy ovaries contain abnormal masses of luteal tissue, derived mainly from atretic follicles, and thus represent a pathologic condition which would seem to have little therapeutic value.

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